The Nociceptive Membrane









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The Nociceptive Membrane

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Current Topics in Membranes, Volume 57

The Nociceptive Membrane

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Foreword

Nociceptive Membrane: Painful Channels in Nociceptors

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A surprising number of ion channels have been discovered in recent years. It is a challenge for researchers to keep up with the flood of information on these new channels. Sometimes, these channels have multiple functions, which further increase the necessary e;ort to determine the purpose of the channels. Most of the recently discovered transit receptor potential (TRP) channels are somewhat linked to sensory transduction in sensory neurons and are also presumed to be linked to nociception. Even though many reviews have been published on these channels in sensory neurons, they are often limited to a class or subfamily of ion channels. Thus, it would be natural to ask if there is a monograph that describes and summarizes ion channels that are expressed in sensory neurons and are suspected to be related to pain.

The preparation of this book involved the collection of almost all the available updated information about ion channels that are linked to pain or nociception. Obviously, TRP channels are introduced in detail due to their role in the generation of nociceptive signals. TRPV1 is discussed in detail because its role in the mediation of inflammatory pain has been intensively studied. In addition, other TRP channels that are sensitive to changes in temperature or those that have been suggested to be related to mechanotransduction are also discussed. Details of ATP-sensitive, P2X and acid-sensing, acid-sensitive ion channels (ASIC) channels are also discussed with great emphasis placed on their possible roles in nociception. It is tempting to think that voltage-gated channels in sensory neurons are less important for mediation of nociceptive signals. However, some subtypes of these voltage-gated channels are di;erentially expressed in many types of pathological conditions, thus implying the importance of their role for pain signals. Furthermore, voltage-gated channels have great potential to modulate or

modify pain signal generation, thus becoming good targets for developing a new class of analgesics.

In addition, this monograph presents the historical background on this pain research field. Dr. Zimmermann gives us a good overview of the historical aspects of establishing the pain concept. Dr. Szolcsanyi and Dr. Willis define nociceptors and introduce a historical overview on studying nociceptors and channels in sensory neurons.

I am extremely grateful to all the contributors for the time and e;ort they have devoted toward their excellent reviews. I have no doubt that this monograph will become a good resource for studying painful channels in nociceptors, owing to the expertise and diligence of the contributors. I also thank Dr. Sid Simon for giving me an opportunity to edit this book and Elsevier for their patience and perseverance toward the publication of this volume for *Current Topics in Membranes*.

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CHAPTER 1

Historical Evolution of Pain Concepts¹

Manfred Zimmermann

Neuroscience and Pain Research Institute, Heidelberg, Germany

- I. Overview
- II. Pain: A Function of Brain Matter in the 17th Century
- III. Physics and Pseudophysics in Pain Treatment of the 18th Century
- IV. Discoveries in the 19th Century Relating to Pain
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 - the 20th Century
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I. OVERVIEW

The purpose of this chapter is to present a short history of pain concepts that emerged in the 17th century, at the time when sensory and motor functions became clearly associated with brain matter rather than the heart or brain ventricles. It was during this period when the background was formed for channels, receptors, and transmitters. Prior to the 17th century, sensations and emotions were mostly thought, for over 2000 years, to arise in the heart and, more recently, in the brain ventricles. The article does not deal

¹This article is adapted from a translation of Zimmermann (2001), with permission.

with medieval or earlier times. Rey (1993) provides an excellent history of pain prior to and overlapping with the dates considered in this chapter.

II. PAIN: A FUNCTION OF BRAIN MATTER IN THE 17TH CENTURY

In the 17th century, the theory of nervous system function was much advanced by René Descartes and Thomas Willis, dramatically changing the prevailing views on pain. Descartes (1596–1650) provided new concepts of the functional anatomy of the sensory organs and brain in his book *De Homine* (published posthumously in 1662). He was the first analytical scientist in neurophysiology, particularly including pain as a sensory-motor system.

The nerves conducting sensations were shown as tubes containing a fluid as the messenger system. Inside the brain these tubes had pores or valves that could open and close in order to control activity of the brain. Here, in response to the sensory input, the same mechanism of opening and closing valves activates the motor nerves inducing the movements to escape the noxious stimulus (Fig. 1). Obviously the valves or pores conceived by Descartes were the universal elements to initiate sensory and motor messages, and thus may be considered the visionary forerunners of modern channel concepts.

For Descartes, the pineal organ was the tentative locus of the conscious perception of a pain stimulus. It was here that the signals of the outer world (*res extensa*) and the level of thinking and imagination (*res cogitans*) became connected to each other.

On the basis of this symbolic presentation, Descartes was considered to have introduced an extremely mechanistic view of nervous system functions. I do not share this obvious criticism because in any modern textbook of neurophysiology, the same basic types of illustrations and interpretations are used to explain brain functions. Among Descartes' great innovations, he recognized for the first time the spatial mapping of the outer world in the brain and the basic principles of the brain's input–output functions. Descartes never claimed to explain mental functions (Procacci and Maresca, 1998).

Thomas Willis (1622–1675) provided overwhelming evidence that the brain substance, including the cortex, is the substrate of sensory functions and not the brain ventricles, as previously believed. For example, he localized the affective dimension of pain in the corpus striatum and corpus callosum, thus anticipating limbic system function and particularly the role of the gyrus cinguli in pain. The motor and visceral reflexes associated with pain are initiated here and conducted via the cerebellum and brainstem, whereas the commands for the expression of pain are conducted to the facial muscles via the pons.



De Rene Descartes

FIGURE 1 Concept of pain processing in the brain by Descartes (1662). Here Descartes visualizes his idea of the brain substance as a fine meshwork, organized to provide the specific and differential functions in pain sensation, motor responses, and autonomic control. In his text description he explains that nerve function is associated with small valves which open and close to release the nerve fluid. Recruiting the valves in open state is a principle of graded sensations and motor strength. The outer space is represented by its orderly mapping of nerves to the brain surfaces. This sophisticated functional concept goes much beyond Descartes' widely reproduced simplistic drawing where he compares the transmission of a pain stimulus with a bellpull operating the doorbell (From Descartes, 1662).

Thomas Sydenham (1624–1689) is known as a great reformer of medicine in England ("the English Hippocrates"). He promoted the consistent and systematic treatment of pain. The analgesic drug laudanum, a composite of opium, saffron, cinnamon, and cloves in wine, was named by him. Based on his own experience as a pain patient, Sydenham strongly promoted laudanum as an indispensable basic tool of every physician. He complained that medicine performed without laudanum is rudimentary. According to him, laudanum was God's gift to console the suffering.

Sydenham gave the first comprehensive description of gout, a disease that he had himself. Apart from promoting the use of laudanum to treat the exacerbation of pain in gout, he also emphasized the role of lifestyle and diet in the pathogenesis and course of the disease. Thus, Sydenham was a pioneer of preventive measures in pain medicine.

The use of opium, consistently promoted by Sydenham, had strong opponents among his contemporaries. One of them was Georg Wolfgang Wedel (1645–1721), professor of medicine at the University of Jena in Germany. In 1684, Wedel published warnings that, among all the medications used by doctors, opium would require the doctor's most serious concern because of its dangerous and potentially lethal effects.

III. PHYSICS AND PSEUDOPHYSICS IN PAIN TREATMENT OF THE 18TH CENTURY

Applications of electricity and magnetism in medical therapy were prominent innovations in 18th-century medicine. Electricity generators using rotating glass disks had already been developed before 1700. Among the pioneers, the German physicist Otto von Guericke (1602–1686) published his experiments in 1672, and the British chemist and physicist Robert Boyle (1627–1691), a founder of the Royal Society (1662), published his research on electricity in 1675. Within a short time, research on the nature of electricity and its propagation and remote actions had become an important topic in physics throughout Europe. This research received considerable support from the academies of science, which provided prizes for progress and discoveries in physics.

A breakthrough in medical use of electricity was initiated by the discovery of the Leyden jar (1745), which could be used for the storage and transportation of static electricity at high voltage. This was a glass bottle coated on the inside and outside with a metalic layer—a forerunner of the electric capacitor, today a major element of electronic technology. The Leyden jar was charged by transiently connecting the metal layers with the poles of the

1. Historical Evolution of Pain Concepts

generator. Several Leyden jars were charged for a treatment session during which they were discharged through the patient's body, mostly at the site of the disease or the pain. Discharges usually were associated with sparks; they evoked muscle and cardiovascular reflexes and sharp and strong sensations, often painful and frightening. This technology echoed the therapeutic use of electric fish known from ancient medicine.

A detailed description of treatment with electrical discharges was published in the 1766 book by Johann Gottlieb Schäffer (1720–1795), whose title translates to *Electrical medicine or the force and effect of electricity on the human body and diseases*. Schäffer was a natural scientist as well as a medical practitioner. He used an electrostatic generator and Leyden jars to treat diseases associated with paralysis and pain (Fig. 2). According to Schäffer's description, the greatest therapeutic effect was obtained with "shaking electricity," when sparks were used to transmit the electricity to the patient, inducing strong muscular twitches, cardiovascular reflexes, and sensations that were usually painful. Schäffer also reported on the medical use of lightning during thunderstorms, which was conducted from the roof into the house with metal wires. Some of these applications had a fatal outcome for the patient or doctor, or both.

Schäffer's book contains impressive case descriptions of electrotherapy in paralysis, arthritis, rheumatism, gout, headache, cramps, and stroke. Some patients were free of symptoms after a single treatment, while others received repeated sessions. Schäffer explained the curative effects of electrical therapy mainly by the increases in blood flow. In addition, he claimed that nervous system functions become reactivated by external electricity, which would facilitate the return of normal function. This is still one of the principles of *electricity* used in neurorehabilitation.

Electrotherapy continued to be used and developed for 250 years. In the late 19th century, Jean-Martin Charcot in Paris used a steam engine to operate an electrostatic generator to administer electrotherapy to psychiatric patients. Today, transcutaneous electrical nerve stimulation (TENS) has become a convenient small-scale version of electrotherapy for pain.

Another highlight of medicine originally associated with physics in the 18th century was presented by Franz Anton Mesmer (1734–1815), who claimed to utilize the physical forces of magnetism for medical treatment (Florey, 1987). Today we know that Mesmer blatantly misinterpreted his observations. Mesmer is now partly credited with the discovery of hypnosis and related psychological forces in medicine, which are of great interest in modern pain medicine.

Mesmer was a student of metaphysics, theology, and medicine. His medical dissertation was on the influence of the planets on the human body. As a medical practitioner in Vienna, he founded the doctrine of "animal



FIGURE 2 Print showing the use of electricity to treat a patient, seen with his doctor in the rear cabinet. Electricity was produced by an electrostatic generator, consisting of rotating disk of glass or other insulating material. The electrical charge was conducted to and stored in Leyden jars (foreground right), which were carried to the patient. Electricity was then discharged to the patient's body via leads guided to the site of the disease (pain or paralysis) by the doctor. Figure reproduced from a facsimile reprint (1977) of Schäffer (1766).

magnetism" and developed this idea into a new therapeutic method. Originally, Mesmer used iron magnets to treat various diseases, meeting with amazing success. He claimed that the therapeutic effect of the magnets *was* transmitted to the patient by the "fluidum," which was hypothesized in

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earlier physics to be the carrier of electrical and magnetic long-range effects. Mesmer soon observed that he could induce curative effects in patients just by directing his hands toward the patients, without using magnetized iron. He concluded that these effects originated from the magnetic forces originating from his own body.

In an attempt to amplify his magnetic forces, he used Leyden jars at room size, termed the "magnetic desk" or "baquet." He hosted ritual séances with groups of patients where he would play the glass harmonica, an instrument producing wonderful magical *sound* much attracted also by Mozart who composed the "fluidum" of physics was now reinterpreted by Messmer as a carrier of cosmic vital forces. The rituals became more and more magical; female patients were particularly susceptible to these ritual acts, and were put into a trancelike state of "magnetic sleep." Physicians and charlatans increasingly used Mesmer's methods worldwide with variations of the magical rituals.

Mesmer was a kind of magician, but he was convinced that he was using scientific principles based on the physics of magnetism. However, Mesmer's methods were increasingly criticized as a pseudoscience, and in 1784 the Societé Royale in Paris rejected Mesmer's method as quackery. He was vindicated in 1812 by a medical committee guided by Wilhelm Christoph Hufeland, a highly respected medical professor in Berlin, and in 1831 the Paris Medical Academy also consented to Mesmer's posthumous vindication. In 1841, James Braid, an English physician, introduced the term "hypnosis" for the new therapeutic method unwittingly introduced by Mesmer.

IV. DISCOVERIES IN THE 19TH CENTURY RELATING TO PAIN

A. Psychophysics of Experimental Pain

In the 19th century, pain became a subject in physiology. Magnus Blix (1849–1904) in Uppsala and Max von Frey (1852–1932) in Würzburg discovered that the spatial fine structure of cutaneous sensitivity consisted of discrete points at which sensations could be elicited by mechanical, thermal, or noxious stimuli (Handwerker and Brune, 1987). They used calibrated hairs and bristles to exert defined mechanical stimuli to the skin of human subjects. The mosaic of pain points was different from that of the touch points. The conclusion from these psychophysical experiments was that specific sensory nerve endings exist in the skin for the sensation of painful stimuli. These "pain points" were associated anatomically with the intraepithelial free nerve endings. Adolf Goldscheider (1858–1935) used the same instruments as von Frey to analyze cutaneous sensations, including pain. However, the conclusions formulated in his "intensity theory" or "summation theory" were at variance with those of von Frey; with increasing intensity of mechanical skin stimulation, the sensations reported by the subjects changed continuously from touch to pain (Goldscheider, 1920). In Goldscheider's view, the neurophysiological information for touch and pain originate from the same set of nerve receptors.

While Goldscheider used the same equipment as von Frey to stimulate the skin, his stimulation paradigm included repetitive trains of stimulation and assessment of transitional phenomena during repetitive stimulation. Also, he included patients with abnormal skin sensations in his studies and accounted for their pathophysiological sensory phenomena when conceiving his summation theory. Goldscheider's observations and interpretations have been of great influence on subsequent pain theories, including the gate control theory (Melzack and Wall, 1965).

B. The Discovery of Ether Anesthesia

The anesthetic effect of nitrous oxide ("laughing gas") was reported as early as 1800 by the English chemist Sir Humphrey Davy (1778–1829), and that of ether was described in 1818 by the English physicist Michael Faraday (1791–1829). Both of these reports mentioned the possibility that anesthesia could be utilized for surgery (DeMoulin, 1974). There has been much discussion among medical historians on why these early reports did not immediately spark experiments with anesthesia for surgery, which was delayed for nearly 50 years!

In 1846 the first surgical operation using ether was conducted by John Collins Warren (1778–1856). The ether anesthesia was performed by William T. G. Morton (1819–1868), a dentist who had used this new type of anesthesia to completely suppress pain sensation during dental extraction. The report of Warren's cancer surgery without pain elicited a rush to use ether to prevent pain in various medical conditions.

In Edinburgh, Sir James Young Simpson (1811–1870) used ether in childbirth as early as in 1847. In the same year he discovered chloroform as an alternative to ether. Childbirth under anesthesia was heavily criticized by Calvinistic ministers, who declaimed that the pain of childbirth was imposed by God on mankind, as stated in the Bible, when Adam and Eve were chased out of Paradise: "In pain shall you bear children."

Ether anesthesia resulted in a wave of opposition. The early comprehensive review on the new method by Dieffenbach (1847; reprinted in 1985) contains a detailed record of its unwanted effects, such as hallucinations, nightmares, cramps, and even death. However, artificially pain-free childbirth was finally accepted when Queen Victoria asked for anesthesia with chloroform in her eighth childbirth in 1853. An advanced chloroform inhaler as used in that time is shown in Fig. 3.



FIGURE 3 Chloroform inhaler as developed and used by John Snow, Queen Victoria's anesthetist at her eighth childbirth (1853). The mouth and nose adapter was equipped with an expiration valve. The sectional drawing of the evaporator shows air being forced through the chloroform compartment. Figure from the book by John Snow, *On Chloroform and Other Anesthetics* (National Library of Medicine, Bethesda, MD, 1858). Reproduced from Lyons and Petrucelli (1978).

The French physiologist François Magendie (1783–1855) opposed the use of ether in the Academy of Science in Paris. He strongly rejected the deathlike state that enabled doctors to cut and scrape unrestricted by the patient's pain. Magendie's argument was that pain is one of the strongest driving forces of life that simply should not be removed. In addition, Magendie warned of the dangerous exacerbation of sensuality, comparable to the effects of animal magnetism, which would place women in particular at a risk similar to that of inebriation.

C. The Discovery of Local Anesthesia

Another sensational event, following the discovery of ether anesthesia, was the discovery of local anesthesia by Carl Koller (1857–1944) in Vienna. Koller used cocaine, a plant alkaloid extracted from South American coca plants. Cocaine, well known as a psychotropic substance, was traditionally used as an energizer by the Incas living at high altitudes in the Andes. An alcoholic extract from coca leaves became fashionable as a stimulating drink under the label "Vin Mariani" in France.

Sigmund Freud (1856–1939) in Vienna investigated the performanceenhancing and psychotropic effects of cocaine; he alerted the young ophthalmologist Carl Koller to the numbness that could be induced by cocaine in the oral cavity. Koller was aware of the need of a local anesthetic substance to perform invasive treatments on the eye that would prevent pain and disturbing reflexes. Although a publication by the physiologist von Anrep had appeared in 1879 in *Pflügers Archiv der gesamten Physiologie*, describing cocaine's local anesthetic effects in animal experiments, the enormous practical significance of this finding had remained unnoticed for several years.

Koller started experiments on animals and on himself and his colleagues, and within a short time he had evidence for rapid and complete local anesthesia in the eye. The first clinical application was a cataract operation under irrigation of the eye with a 2% cocaine solution, an operation that was completely pain free. A few days later, in September 1884, he asked a colleague traveling to the German Ophthalmology Congress in Heidelberg to present and demonstrate his findings, as he lacked the funds to travel to Heidelberg himself. In the same year, Koller (1884) published his discovery in *The Lancet*. Koller's biography was published 80 years later by his daughter, with reference to many important documents relating to the discovery and the broad foundation of local anesthesia in medicine (Koller-Becker, 1963).

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D. Isolation and Synthesis of Analgesic Substances

Some basic analgesic drugs for systemic use were also discovered in the 19th century: morphine, aspirin, antipyrine, and phenacetin.

1. Sertürner and Morphine

In 1806, the young pharmacist Friedrich Wilhelm Sertürner (1783–1841) reported on his discovery and extraction of the sleep-inducing substance (*principium somniferum*) contained in opium, the extract of poppy juice used in medicine for centuries. He tested his extracts in heroic experiments on himself and his friends (Fig. 4).

In those times pharmacy was not an academic profession but was learned in a 4-year apprenticeship training. Sertürner completed this apprenticeship and passed a final examination to obtain the qualifications necessary to conduct a pharmacy. He started his experiments on opium soon after completion of his training at the age of 20 years. The publication of his discovery (Sertürner, 1806) remained unnoticed. He changed the subjects of his scientific interest, working on alkali and electricity. After nearly 10 years he resumed his investigation of opium extracts and used the new label "morphium" (derived from Morpheus, the Greek god of sleep) for the sleep-inducing substance (Sertürner, 1817). This time his publication was widely acknowledged and Sertürner became famous throughout Europe. The University of Jena awarded him the degree of Dr. Phil. in his absence, acknowledging the 1817 publication as his doctoral thesis. Subsequently, several other investigators, in particular from France, claimed priority in the discovery of morphine. These controversies were terminated in 1831 when the Institut de France awarded Sertürner a prize of 2000 francs for his discovery of morphine.

The production of morphine was taken over by another pharmacist, Heinrich E. Merck in Darmstadt (Germany), starting in 1820. The laboratory of Merck's pharmacy was the origin of the E. Merck Company in Germany as well as Merck, Sharp, and Dohme in the United States.

2. Development of Aspirin, Metamizol, and Paracetamol (acetaminophen)

Later, several other plant substances, which were traditionally used to alleviate pain, became the targets of drug development. The rationale was to identify and isolate the active molecule in a plant extract.

The Italian chemist Raffaele Piria (1815–1865) isolated salicylate, a glycoside, from willow bark, which had long been used as an analgesic. Salicylic acid was derived as a synthetic product from salicylate, the sodium salt



Journal der Pharmacie For Aerzte, Apotheker und Chemisten von D. Johann Bartholmä Trommsdorf. Vierzehnter Band. Leipzig 1806.

Darstellung der reinen Mohnsäure*) (Opiumsäure) nebst einer

chemischen Untersuchung des Opiums

mit vorzüglicher Hinsicht auf einen darin neu entdeckten Stoff und die dahin gehörigen Bemerkungen.

> Vom Herrn Sertürner in Paderborn.

Im Journale der Pharmazie 13ten Bandes machte ich einige Bemerkungen über die besonderen Eigenschaften des im Handel vorkommenden Opiums, welche mir nach den bis jetzt bekannten Bestandtheilen desselben unerklärbar waren; auch äußerte ich zugleich, daß jene Erscheinungen von einer eigenen noch unbekannten Säure determinirt würden, ohne jedoch einen entscheidenden Beweis dafür anzugeben.

An eben erwähntem Orte machte deswegen Herr Professor Trommsdorff nicht ohne Grund die Bemerkung, daß es meinen Versuchen zufolge zweifelhaft sey, ob jene Phänomene von einer Säure oder einem anderen Stoff bewirkt würden; es war sehr zu

*) Dieses scheint wir der angemessenste Name zu seyn, weil ich sie bis jetzt in keinem andern Vegetabil als dem Mohne gefunden habe. Fr. Sertürner 3

FIGURE 4 Friedrich Wilhelm Sertürner, the discoverer of Morphine. Lefthand, Sertürner's portray, taken between 1830 and 1840, *Lithograph* by Julius Giere. Righthand, Starting page of Sertürner's publication of 1806 describing Opium acid and the extraction of "sleeping substance" from Opium. In his later publication (1817) he replaced "sleeping substance" with "Morphine" for the first time.

which was produced industrially and became widely used from 1896 in Germany for the treatment of fever, polyarthritis, and sciatica (Handwerker and Brune, 1987; Havertz *et al.*, 1996). The final stage in the refinement of this drug was its acetylation, invented in 1897 by Felix Hoffmann, a chemist in the laboratory of the Bayer Company. The resulting acetylsalicylic acid, a powerful analgesic, was marketed by Bayer from 1899 under the label Aspirin. Aspirin was the first drug to be protected by a patent, and it remained the front-runner of analgesic drugs for more than 100 years.

Other synthetic analgesics were discovered in the search for drugs against fever. The first compound of the chemical group of pyrazolinones was antipyrine, jointly developed in 1884 by the chemist Ludwig Knorr and the pharmacologist Wilhelm Filehne at the University of Erlangen, followed by aminopyrine (Pyramidon) in 1896. A pyrazolone drug still used today is the sodium sulfonate of aminopyrine (known as metamizol sodium in Europe and as dipyrone in the United States and Britain).

The aniline derivatives, with acetanilide a prototype, also were originally developed as antipyretic drugs in 1887. However, their excellent analgesic effect soon became evident, changing the primary indication of these drugs to the treatment of pain. Phenacetin, most widely used as an over-the-counter analgesic, turned out to be nephrotoxic with prolonged use, and therefore was withdrawn from the market. Paracetamol (known as acetaminophen in the United States) was identified as an analgesic metabolite of phenacetin by Julius Axelrod in New York (1948) and became available as an analgesic drug in 1956.

Facsimile reprints of key publications in German relating to the development of some of these drugs were published, together with an English translation, on the occasion of the 5th World Congress on Pain in Hamburg in 1987 (Handwerker and Brune, 1987). They provide a direct glimpse into the pioneering era of analgesic drug development.

E. Recognizing Neuropathic Pain in a War

Great progress in the understanding of neuropathic pain was achieved by Mitchell (1872) during the American Civil War. As had happened in the preceding centuries, war imposed major challenges on physicians to treat painful wounds. From 1863, Mitchell, a neurologist, provided detailed descriptions of posttraumatic neuralgias, phantom pain, and causalgias from cases he treated at the Philadelphia Hospital. For example, he observed patients with extreme hyperalgesia (or allodynia, according to modern taxonomy) following gunshot lesions of major nerves to the arms and legs. In some of these patients, severe amplification of burning pain occurred during excitement associated with sympathetic cardiovascular activation. Mitchell was the first to use the term *causalgia* for this syndrome, and he introduced innovative treatments including ice packs and baths, as well as bandages to protect against touch stimuli.

In the case of continuous excruciating pain, Mitchell routinely administered morphine injections several times a day. He administered a total of 40,000 morphine dosages within 1 year, and some patients received up to 500 injections per year. Mitchell reported on the excellent analgesic effect of the morphine. He administered morphine locally to the site of the lesion in cases of causalgia and therefore may be credited for the first observation of a peripheral opioid action.

Apart from his enthusiastic descriptions of the analgesia elicited by morphine treatment, he also reported on "very unfavorable physical and moral conditions" related to the use of morphine, that is, the induction of dependence and addiction. Today he could have prevented such unfortunate outcomes by administering morphine at regular time intervals, following the guidelines introduced by the World Health Organization in 1986.

V. CHALLENGES AND CHANGING PAIN CONCEPTS DURING THE 20TH CENTURY

The first half of the 20th century left millions of patients in pain from injuries sustained in two world wars. Enormous new challenges arose with the need for treatment and care of those with old and new pain syndromes, and with increasing needs for medical education. After World War II, anesthesiologists and neurosurgeons were the first to respond to these challenges, resulting in rapidly increasing therapeutic skills and newly developed scientific concepts related to pain. First, I will review the theoretical background that developed during the early 20th century.

A. Clinical Research on Pain

Sir Henry Head (1861–1940), a neurologist in London, criticized the specificity theory of skin sensation as formulated by Max von Frey (described in an earlier section) because it was inadequate to explain clinical pain cases. Head used a different approach based on his clinical observations and his experience of sensory alterations following self-inflicted transections of some of his cutaneous small nerve branches. Accordingly, he formulated a new theory of the somatosensory system, outlining the *epicritic* and *protopathic* sensitivities of somatosensation (Head *et al.*, 1905). Epicritic

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sensitivity referred particularly to well-localized touch sensations, while protopathic sensitivity referred to poorly localized, dull, disagreeable, and pathological sensations, which were, for the most part, related to the realm of pain sensations with a strong affective quality. According to Head, protopathic sensitivity was the first to reappear in the case of a regenerating nerve.

On the basis of electrophysiological studies by Erlanger and Gasser (1937), epicritic sensitivity was associated with the fast-conducting, largediameter, myelinated A fibers in peripheral nerves. Protopathic sensitivity, on the other hand, was associated with the slowly conducting, nonmyelinated C fibers. An important basic observation by Head was that protopathic sensitivity was inhibited or masked by simultaneous activation of epicritic sensation. Later researchers found inconsistencies in Head's conception of a dual mechanism for cutaneous sensation, and it was nearly abandoned. In the context of neuropsychological investigations with brain imaging, interest is reemerging in Head's conceptualization of these dual aspects of sensation.

Earlier work by Head (1893) was related to pain referral from the viscera to the body surface. The skin areas to which visceral sensations are referred were soon termed *Head's zones*, now explained physiologically by the convergence of visceral and cutaneous afferent fibers onto a pool of spinal cord neurons. Head's zones and other signs of pain referral remain basic principles in the diagnosis and assessment of internal diseases.

Otfried Foerster (1873–1941), a neurosurgeon and psychiatrist in Breslau, Germany, exploited therapeutic neurosurgery to study the human nervous system. He was one of the first surgeons to systematically use cordotomy (transection of the spinal anterolateral tract) for the treatment of pain. He carefully mapped the human dermatomes of therapeutic cases using spinal root transection or electrical stimulation. His clinical experience, enriched by ingenious conceptualization, was published in a valuable monograph on central pathways of pain (Foerster, 1927), which is still essential reading material for the pain scientist and clinician. For example, he concluded from some clinical observations that spinal sensory transmission is under descending control from the brain.

The concepts of pain based on clinical studies by Head, Foerster, and others were further elaborated by William Noordenbos (1959) into a pain theory of central interactions, and his book *Pain* was a major support for the gate control theory (Melzack and Wall, 1965).

René Leriche (1879–1955), a French surgeon, became a provocative and emphatic advocate of pain therapy in his lectures at the Collège de France, published in his book *Chirurgie de la Douleur* (Leriche, 1936). He coined the concept of pain as a disease (*douleur maladie*) to which multiple factors contribute. He emphasized excruciating chronic pain related to dysfunction of the sympathetic nervous system, and therefore he recommended interventions on the sympathetic nervous system. He criticized the overemphasis on pain as a tool in medical diagnosis, which often resulted in insufficient pain treatment and unnecessary suffering. He also criticized the fact that patients complaining of excruciating pain were often considered neurotic by doctors.

B. Experimental and Clinical Neurophysiology of Pain

Yngve Zotterman (1898–1982), a Swedish neurophysiologist, had recorded the first single nerve fiber action potentials in Lord Adrian's laboratory in Cambridge in 1926. Ten years later, Zotterman (1936) published the first action potentials recorded from a single nociceptive nerve fiber, probably a nonmyelinated C fiber, in the cat's tongue in response to noxious heating (Fig. 5). He was the first to use, in pain research, the cathode ray oscilloscope, which had just been introduced in neurophysiology (Erlanger and Gasser, 1937). From the 1950s, the functional characteristics of nociceptors were systematically explored in Edinburgh by the neurophysiologist Ainsley Iggo (born in 1922).

In the 1930s and 1940s, several contributions were published that became important for the development of pain concepts relevant to clinical phenomena. Thomas Lewis (1881–1945), a doctor of internal medicine in London, published his studies on pain, mostly conducted on human subjects and

Polymodal nociceptor in the lingual nerve of the cat



FIGURE 5 First electrophysiological records of unitary action potentials from a single nociceptor in the cat's lingual nerve. The receptive field of this nerve fiber was on the tongue of the animal and could be excited by heavy pressure (upper record) or hot water at 60° C (lower record). The large action potential is from a low-threshold mechanoreceptor responding to the pressure stimulus as well, the small action potential is from the nociceptor. Reproduction from Zotterman (1936).
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patients, in a stimulating monograph (Lewis, 1942). His themes were visceral pain, referred pain, and primary and secondary hyperalgesia. He postulated the existence of a humoral nocifensive system based upon chemical mediators that slowly increased pain sensitivity in the environment of a primary lesion. Among the mediators he identified were histamine, bradykinin, and substance P. From his discoveries the concept of neurogenic inflammation emerged, which is now considered to contribute to pain-enhancing interactions between the nervous and immune systems in rheumatic and neuropathic diseases.

Innovative studies on the phenomenon of hyperalgesia were performed at Yale University by James D. Hardy (a physiologist) and Harold G. Wolff (a neurologist) on human subjects and patients (Hardy *et al.*, 1950). These investigators identified central mechanisms in secondary hyperalgesia as mediators of progressively painful diseases. They built their ideas on a "neuronal pool" of variable excitability in the spinal cord, which had been conceptualized in 1943 by the surgeon William K. Livingston (1892–1966). His monograph (Livingston, 1943) is still a source of ideas on pain mechanisms. Livingston treated and studied complex clinical pain phenomena during World War II, such as referred pain and causalgia, and emphasized their continual exacerbation if they were not treated consistently. In 1947, he initiated a pain project at the University of Oregon to study the physiology and psychology of pain in a clinical setting.

The concepts developed in about 1950 by Hardy, Wolff, Livingston, and others, relating to spinal mechanisms of hyperalgesia, were rediscovered and extended from about 1985 onwards, and now form part of our understanding of the central components of hyperalgesia and the progressive chronicity of pain.

C. New Vistas on Pain Since 1950

After World War II, enormous challenges arose relating to the medical care of the millions wounded during the war. The foremost pioneer of their care was John Bonica (1917–1994) in the United States, who established the first interdisciplinary pain clinic in 1947 to treat pain problems of wounded veterans. This clinic became part of the University of Washington in Seattle in 1960 and received worldwide recognition for its interdisciplinary treatment of pain problems. Bonica's *Management of Pain* (now in its third edition) became a bible for generations of doctors interested in pain treatment (Bonica, 1953). It was Bonica's initiative and efforts that resulted in the foundation of the International Association for the Study of Pain (IASP) during the unforgettable International Symposium on Pain in Issaquah, Washington, May 21–26, 1973 (Bonica, 1974).

At the same time there was renewed interest in the basic science of pain. The publication of the gate control theory by Melzack and Wall (1965) was aimed at an integration of the views of neurophysiology, psychology, and the huge variety of clinical phenomenon related to pain in patients, in an attempt to set up a coherent system of ideas. Although some of its neurophysiological details were later disproved (Zimmermann, 1968), the gate control theory provided a new perspective on pain for medical scientists and is now considered the foundation of modern conceptions of pain.

Since then, there has been great progress in all aspects of pain research. The most important milestones were the discovery of endogenous opioids and central nervous system pain control systems, research on the neuro-modulation of pain, oral and spinal administration of opioids for chronic pain, and work on psychological elements in chronic pain and pain therapy. Other important topics have emerged, including the plasticity of the nervous system and progressive pain chronicity, risk factor models for pain, genetic determinants of pain and pain treatment, and interdisciplinary networks in the health system for the therapy and prevention of pain.

I wish that all of this enormous progress in the knowledge on pain will help to better manage pain as a major health problem.

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CHAPTER 2

History of Ion Channels in the Pain Sensory System

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- VII. Initiation of Impulses at Nociceptors References

I. INTRODUCTION

A century ago Sherrington (1906) postulated the existence of a distinct class of sensory nerve endings, which respond to tissue damaging or potentially injurious (noxious) stimuli suitable for evoking pain sensation in humans or protective reflexes in animals. For labeling these sensory nerve terminals he coined the term "nociceptors." Early studies of recording from myelinated afferents (Adrian, 1926: Zottermann, 1933) or from unmyelinated single C-afferent fibers (Iggo, 1959: Iriuchijima and Zotterman, 1960) of peripheral nerves provided examples for sensory receptors, which respond to hot or tissue damaging mechanical stimuli. The rare occurrence of these high-threshold sensors, however, markedly contrasted with the numerous types of low-threshold mechanoreceptors identified until the first half of 1960s. It was due to technical difficulties for recording single unitary discharges from unmyelinated or thin myelinated fibers. Nevertheless, these data formed the cornerstone of challenging the concept of the existence of nociceptors (Melzack and Wall, 1965). Subsequent thorough single unit studies on large number of A δ and C-afferent fibers provided unequivocal evidence for the existence of nociceptors and led to the discovery of $A\delta$ mechanonociceptors (Burgess and Perl, 1967) and C-polymodal nociceptors (Bessou and Perl, 1969). The latter group of cutaneous afferents was excited by noxious heat, mechanical stimuli and some irritants such as acids applied to the unbroken skin. It forms the largest group of cutaneous C-afferents (30 -100% of fibers) in different nerves of the rat, rabbit, cat, pig, monkey, and humans (Lynn et al., 1995; Lawson, 1996), and in the mouse where 68% of them were excited by both heat and cold stimuli (Cain et al., 2001). In further studies response characteristics of these nociceptors to noxious physical stimuli were described in detail (Belmonte and Cervero, 1996; Kumazawa et al., 1996). However, their selective excitability by chemonociceptive stimuli remained elusive. On one hand topical application of irritants to unbroken skin or onto the blister base (Foster and Ramage, 1981) supported the selective chemical excitability of cutaneous C-polymodal nociceptors. In internal organs, similar chemonociceptive polymodal receptors conducting with A δ and C-fibers were also described (Kumazawa *et al.*, 1996). On the other hand intra-arterial injection of bradykinin and serotonin (Beck and Handwerker, 1974) or in vitro superfusion of the subcutaneous side of the rat skin with bradykinin (Lang et al., 1990) activated also nonnociceptive types of cutaneous afferents, raising the possibility that in the skin the superficial position of C-polymodal nociceptors and not their unique excitability spectrum is responsible for their chemonociceptive nature. In further studies, however, it turned out that a low dose of bradykinin (0.2 µg) given intraarterially into the ear artery of the rabbit elicited discharges exclusively in

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single unit C-polymodal nociceptor fibers, while in a 10 times higher dose other types of receptors were also excited (Szolcsányi, 1980, 1987; Perl, 1996). Thus, the high-threshold excitability of C-polymodal nociceptors to thermal and mechanical stimuli is combined with a low-threshold excitability by endogenous pain-producing chemical agents, indicating that these nerve terminals are richly supplied with ligand-gated receptors for various agents.

Capsaicin, the pungent agent of chili peppers has opened up new horizons in the field of physiology, pharmacology, and molecular biology of polymodal nociceptors. This pungent agent led to the discovery of the first temperature-gated ion channel suitable for detecting noxious heat and noxious chemical signals in an integrative way. With the aid of capsaicin, a novel ion channel from dorsal root ganglia was identified, cloned, and termed the capsaicin receptor or vanilloid receptor type 1 (VR1) (Caterina *et al.*, 1997). It was renamed by the Nomenclature Committee of the International Union of Pharmacology to "transient receptor potential vanilloid" type 1 (TRPV1) (Clapham *et al.*, 2003). Adopting the Sherringtonian concept for nociceptors to the molecular level, the TRPV1 capsaicin receptor/ion channel serves as a nociceptive transducer membrane protein.

The present chapter is devoted to shedding light on early discoveries, which led to our present knowledge of different cation channels suitable for triggering nociceptive signals to the central nervous system. Several findings on transfected cell lines or sensory neurons in tissue culture helped us to clarify delicate molecular mechanisms at the cellular level. The nociceptive membrane at the sensory nerve terminals, however, cannot be so well analyzed. These plasma membrane transducer proteins synthesized in the cell body are trafficking to the peripheral nerve terminals, but it should be kept in mind that albeit the same ion channel is present in the soma and in the nerve terminal, their operation and distribution in intracellular membrane structures at the nerve ending may differ from those analyzed in the cell body *in vitro*. Therefore, particular attention was paid to data obtained by single unit recordings from peripheral nerves and those aspects that are not discussed in other chapters. At the end of this chapter a concept is presented about the function of cation channels in the plasma membrane of the nociceptors.

II. THE TRPV1/VR1 CAPSAICIN RECEPTOR

A. Discovery of Capsaicin Desensitization

Capsaicin has become a similar lead molecule for sensory pharmacology like the classical alkaloids of curare, nicotine, ergotoxin, and atropine at the efferent side of the peripheral nervous system. Although some pharmacological actions of capsaicin on sensory systems were known for a long time (Hogyes, 1878), the real starting point of the capsaicin saga was an unexpected observation made by Nicholas (Miklós) Jancsó about 50 years before the capsaicin-gated ion channel was cloned (Caterina et al., 1997). He described that during inflammation histamine served as a physiological activator of the "reticuloendothelial cells" (Jancsó, 1947). In these studies he treated mice and rats with high histamine doses to achieve "histamine desensitization" in order to reveal how significant is the role of this agent in inflammatory processes. In the course of these experiments he used also capsaicin and observed that by repeated instillation of capsaicin into the eye of mice, rats, and guinea pigs a unique type of "desensitization" ensued against "defensive reflexes" evoked by the "strongest known chemical stimuli" like chloracetophenone, potassium chloride, formalin, veratrine, or nicotine (Jancsó, 1955, 1960). It was remarkable, however, that after "capsaicin desensitization" and unlike after local anesthesia responsiveness of the animals remained intact to tactile, noxious mechanical, or noxious heat stimuli.

During his last 4 years before his demise in 1966 one of the authors (J.S.) joined the Jancsó couple, and this fruitful period of capsaicin research led to five posthumous papers that appeared under his name for the first time in high-ranking international journals and in a conference volume on pain (Jancsó *et al.*, 1967, 1968; Jancsó-Gábor *et al.*, 1970a,b).

Major results described in these papers were as follows:

1. Capsaicin, mustard oil, xylene, and chloracetophenone elicit neurogenic inflammation, which is absent after chronic denervation or after systemic or local "capsaicin desensitization."

2. The stimulatory and desensitizing effects of capsaicin take place at the level of sensory receptors. Desensitization by repeated instillations into the eye of the rat can be induced only by pungent acylamides (capsaicin or piperine) but not with the non-acylamide vanilloid zingerone or other irritants like mustard oil.

3. Neurogenic inflammation was evoked by antidromic electrical stimulation of sensory nerves, and the response was absent after local or systemic capsaicin or piperine "desensitization."

4. The local neurogenic inflammation evoked by capsaicin, mustard oil, or chloracetophenone was not inhibited by local anesthetics indicating that its mediator is released from the nerve endings without the intervention of axon reflexes. The same holds true for the human skin where lidocaine did not inhibit the redness and slight edema at the site of contact of a piece of paprika, although the flare was abolished in this way. 5. Systemic treatment with capsaicin or its vanilloid or nonvanilloid congeners and piperine cause hypothermia followed by impairment of thermoregulation against warm but not cold environment.

6. Capsaicin injected into the preoptic area of the rat elicits a fall in body temperature, local desensitization, and these intracerebrally desensitized rats could not cope with overheating of their bodies. In rats pretreated with high s.c. doses of capsaicin, the hypothermic effect of preoptic injection of capsaicin or local heating of the preoptic area was strongly inhibited. It has been concluded that capsaicin desensitizes the "hypothalamic warmth detectors" against capsaicin and their natural stimuli.

These early results with a full list of references of capsaicin research up to the end of 1970s were summarized in detail (Szolcsányi, 1982, 1984a) and afterwards their scopes for further interest were discussed in several reviews (Fitzgerald, 1983; Russell and Burchiel, 1984; Buck and Burks, 1986; Szolcsányi, 1990, 1991, 1993, 1996a,b, 2002, 2004; Holzer, 1991; Maggi, 1995; Szállási and Blumberg, 1999).

B. Prediction of the Capsaicin Receptor on C-Polymodal Nociceptors

The cellular mechanisms of the highly interesting acute and long-term actions of capsaicin on nociception, inflammation, and thermoregulation remained puzzling when Nicholas Jancsó left us. How selective is its effect among sensory receptors? Is the loss of chemical excitability after capsaicin treatment restricted to nerve endings which mediate nociception or do other sensory nerve endings also lose their chemosensitivity leaving their responsiveness to physical stimuli intact? What is the cellular background of the extremely long-lasting loss of responsiveness? In order to obtain information about the effect of capsaicin on different qualities of sensations, Szolcsányi performed self-experiments from the late 1960s on his and his coworkers' tongue and blister bases before and after capsaicin desensitization. Furthermore, the temperature-dependence of capsaicin-induced burning sensation was analyzed on the human skin (Szolcsányi and Jancsó-Gábor, 1973; Szolcsányi, 1976, 1977).

1. Sensory Effects of Capsaicin in Humans: Early Observations

On the tongue, three desensitizing procedures were done in self-experiments by putting the tongue into a 1% capsaicin solution 10 times for 1 min (Szolcsányi, 1977). Control values of recognition thresholds to capsaicin $(2 \times 10^{-7} \text{ g/ml})$, mustard oil $(2 \times 10^{-5} \text{ g/ml})$, zingerone $(1 \times 10^{-3} \text{ g/ml})$ as well

as taste stimuli of quinine sulfate, NaCl, glucose, and ascorbic acid were determined similarly as the tactile threshold and difference limens to tactile and temperature stimuli. Two hours after the treatment was completed, no sensation was evoked by capsaicin $(1 \times 10^{-3} \text{ g/ml})$ or zingerone $(1 \times 10^{-1} \text{ g/ml})$ and a high concentration of mustard oil $(1 \times 10^{-3} \text{ g/ml})$ was felt as sweet. On the other hand cool sensation evoked by menthol or different taste sensations remained unchanged, and thresholds and difference limens to tactile stimuli as well as pain evoked by pinprick or pinching the tongue were not altered. Temperature difference limens in the cold range of 20-25°C remained unchanged, while in the warm range of 36-45°C the temperature discrimination threshold was significantly higher for 7-8 h. Sensory effects of pungent agents returned slowly to the control level within 2 days. These data provided strong evidence for the neuroselective action of capsaicin on chemonociceptive nerve endings since the chemical excitability of other receptors (cold, taste) remained intact. Subsequently these chemonociceptive sensors were identified as the C-polymodal nociceptors (Szolcsányi, 1976, 1977).

The pain induced by capsaicin and endogenous compounds, such as bradykinin, acetylcholine, and KCl, was tested in seven exposed bases of cantharadine blisters (Keele and Armstrong, 1964) before and after application of a 1% capsaicin solution to the blister base for 3-5 min. After this treatment the pain induced by KCl solution remained unchanged, while threshold concentrations of capsaicin, bradykinin, and acetylcholine to induce pain were markedly increased. The lack of effect of capsaicin desensitization on KCl was explained by the KCl-induced axonal excitation of all types of afferents including the mechanonociceptive fibers.

In two further series of experiments the nonlinear temperature-dependence of the excitatory effects of capsaicin on nociceptors was revealed with the aid of sensory assessments on humans and by recording action potentials from the saphenous nerve of the rat (Szolcsányi, 1976, 1977). In Fig. 1, measurements of a series of temperature discriminations of one out of eight tests made on four subjects of the laboratory staff are shown. The dorsal skin of one hand was smeared with 1% capsaicin solution in ethanol, and the other hand was similarly treated with ethanol only. A few minutes later burning pain sensation developed in the capsaicin-treated skin, and if the back of this hand was immersed into a water bath at 31°C it was felt hotter than 45°C applied to the control hand. This burning sensation was accompanied by a pronounced tactile allodynia. However, if the capsaicin-treated hand was put into a cool water bath of 17-28°C both the burning pain sensation and tactile allodynia disappeared within 1-2 s and no impaired temperature discrimination was observed between the two hands. Thus, there was a clear threshold temperature for pain sensation around 30-31 °C, which shifted to a higher level after 16-18 h (Fig. 1). Capsaicin-induced heat

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FIGURE 1 Shift of the threshold for thermal pain in a human subject. Measurements of temperature discrimination ability between the two hands. \bigcirc , control values; \triangle , from 20 to 120 min after one of the hands was painted with a 1% capsaicin solution in alcohol; \Box , 16–18 h after the treatment. Open symbols, distinguishable differences and solid symbols, mistakes or indistinguishable differences. Reprinted from Szolcsányi (1977).

sensation has become a useful human experimental pain model (Petersen and Rowbotham, 1999).

Figure 2 shows that this temperature-dependent sensation is due to a similar thermodependence of the capsaicin-induced excitation of the nociceptors. Frequencies of action potentials recorded from the saphenous nerve of four anesthetized rats are shown in the figure, where A, B, C show the effect of s.c. injection of capsaicin (2 μ g/20 μ l) under the dorsal skin of the hind paw. The local temperature over the skin area was controlled by flowing water. It is striking that at a skin temperature of 20°C (Fig. 2A) capsaicin injection did not evoke discharges, while enhancement of the skin temperature by few centrigrades elicited a burst of action potentials, which was slowly blocked again by cooling the skin area back to 20°C. These recordings clearly show that there were thresholds for firing, which moved upwards during the time course. In Fig. 2C enhancement of the skin temperature from 20°C to 30°C



FIGURE 2 Frequency of action potential discharges of the saphenous nerve of four (A, B, C, and D) rats. Dotted line, skin temperature; \bigtriangledown , introduction of the needle under the skin; \bullet , tactile stimuli occasionally accompanied the maneuvres of changing the temperature; \uparrow , capsaicin injection (2 µg in 20 µl) to the temperature-controlled receptive field of the dorsal skin of the hindpaw. At panel C, capsaicin was injected 10 min before the first mark on time scale. D, control. Reprinted from Szolcsányi (1977).

did not increase the background activity, while increasing it to 35° C elicited definite excitation of the nociceptors. A similar shift in thermal threshold of C-polymodal nociceptor unitary discharges was observed in the human skin after topical capsaicin application (Konietzny and Hensel, 1983). This threshold phenomenon, however, was not detected in cell culture experiments or on TRPV1-transfected cell lines (Voets *et al.*, 2004). Discrepancies between these *in vivo* results and *in vitro* observations will be discussed later in Section VII.

2. Selective Effect of Capsaicin on C-Polymodal Nociceptors

The first electrophysiological evidence for a selective action of capsaicin on C-polymodal nociceptors was obtained with the aid of the collision technique (Douglas and Ritchie, 1960) during the 1970s (Szolcsányi, 1976,

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FIGURE 3 Compound action potentials recorded from the saphenous nerve of the cat before (C) and at different intervals after 20 μ g of capsaicin was given close arterially into the skin area. Upper records show C potentials, lower records A potentials. Each trace was obtained by superposing eight successive sweeps. Tactile stimuli were applied when the last record of A potentials was taken. Reprinted from Szolcsányi (1976).

1977). Figure 3 shows compound action potentials recorded from the cat's saphenous nerve. In this preparation (Douglas and Ritchie, 1960; Douglas *et al.*, 1960; Martin and Manning, 1969), orthodromic action potentials conducting in the C-fiber range collide with the antidromic compound action potentials, which manifest themselves in two waves, C_1 and C_2 . Tactile stimuli or cooling the skin area elicits collision exclusively of the C_1 wave (Douglas *et al.*, 1960), while noxious radiant heat collides with only the C_2 wave (Martin and Manning, 1969). Close-arterial injection of capsaicin in a 100-fold dose range collided exclusively the C_2 wave (upper row) while the $A\alpha\beta$ and $A\delta$ peaks (lower row) and C_1 wave were not diminished.

Single unit studies reported during the 1980s provided further evidence for the selective action of capsaicin on C-polymodal nociceptors (Szolcsányi, 1980, 1987; Foster and Ramage, 1981; Kenins, 1982; Konietzny and Hensel, 1983; Szolcsányi *et al.*, 1988). For reference to more recent single unit studies on the action of capsaicin on afferent fibers see Szolcsányi (1993, 1996a,b, 2004), Belmonte and Cervero (1996), Kumazawa *et al.* (1996), and Szállási and Blumberg (1999).

Close arterial injection of capsaicin (20 μ g) into the main artery of the rabbit initiated with 1–3 s latency unitary discharges in almost all (25/26) C-polymodal nociceptors. None of the tested C-fiber low-threshold mechanoreceptors (C-LTM), high-threshold mechanoreceptors (C-HTM), C-cold receptors or A δ fiber high-threshold mechanoreceptors (A δ -HTM), D-hair receptors, $A\alpha\beta$ G-hair or field receptors was excited by capsaicin over a 100fold dose range (Szolcsányi, 1980, 1987). Close-arterial injection of capsaicin into the femoral artery of the rat showed that it excited not only the C-polymodal nociceptors (18/18), but also the $A\delta$ mechano-heat-sensitive ($A\delta$ -polymodal) nociceptors (4/5). Again, none of the C-LTM, C-HTM, C-cold, $A\delta$ -cold, $A\delta$ -HTM receptors was excited (Szolcsányi *et al.*, 1988). Two out of 10 slowly adapting mechanoreceptors (SA-I, SA-II) responded also to capsaicin but only after a long latency of 10–13 s indicating that some of these highly sensitive mechanoreceptors responded probably to vasodilatation and not to capsaicin *per se*. In rats pretreated by high systemic capsaicin doses the proportion of C-polymodal nociceptors was decreased, C-mechanoreceptors increased, while no significant difference was observed in the proportion of C-cold receptors (Szolcsányi *et al.*, 1988). Thus, among the C-afferents only the subgroup of C-polymodal nociceptors was diminished in rats pretreated with systemic capsaicin in adult rats.

Topical application of capsaicin also induced selective excitation of the cutaneous C-polymodal nociceptors in the cat, rat, and human. In the cat the blister base model was used. In order to evoke a blister, contact heat of 100°C was applied on the skin 2 days before testing. Under these conditions 10^{-4} M capsaicin evoked low-frequency discharges after a long latency (Foster and Ramage, 1981). Mechanoreceptors (C-M, $A\delta$ -HTM, slowly adapting and rapidly adapting A δ -HTM and A $\alpha\beta$ -LTM (slowly adapting, rapidly adapting) were not activated. In another early study on the rat, topical application of 1% capsaicin in 50% DMSO and 50% ethanol was shown to excite selectively the cutaneous C-polymodal nociceptors (Kenins, 1982), but a subsequent more careful analysis showed that part of the action potentials could be attributed to the irritancy of the solvent which was not tested in the former study (Lynn et al., 1992). Nevertheless, results of these papers confirmed and extended the conclusion of Bessou and Perl (1969) that similarly to other irritants, capsaicin and DMSO when applied onto the unbroken skin excite exclusively the C-polymodal nociceptors.

Single unit studies on human, monkey, and pig cutaneous nerves have revealed that intracutaneous injection of capsaicin excites novel subgroups of C-afferents beyond the C-polymodal nociceptors. These units are mechano-heat-insensitive (CM_iH_i) in the human skin (Schmelz *et al.*, 2000), noxious heat-sensitive (C-H) in the skin of the pig (Lynn *et al.*, 1996), and "heat-insensitive" or "mechano-insensitive" A δ afferents in the monkey (Ringkamp *et al.*, 2001). In the latter study a unit was classified as heat-sensitive when it responded to 49 °C and heat-insensitive when a stimulus of 53 °C was required for activation.

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3. Prediction of the Capsaicin Receptor

The selective loss of burning pain sensation with intact chemical excitability of taste and cold receptors discussed before favored a highly selective action of capsaicin on C-polymodal nociceptors. This neuroselective site of action by itself raised the possibility for the existence of a plasma membrane protein, which is expressed in these neurons but not in other sensors. The next hint, which seemed to support the existence of a putative capsaicin receptor, was its species selective action. Instillation of capsaicin into the eye of pigeons or hens or putting frogs into 1% capsaicin solution did not evoke protective reflexes, although in these species other irritants were effective (Jancsó *et al.*, 1967). In a more recent study where capsaicin was injected close-arterially in conscious pigeons the lack of protective reflexes and inability of desensitization with extremely high capsaicin doses (600 mg/kg i.a.) against nociception induced by capsaicin or bradykinin were also shown (Szolcsányi *et al.*, 1986).

In order to develop a capsaicin congener, which is devoid of the excitatory effect leaving the potency for strong "capsaicin desensitization," structureactivity relationships were obtained using a novel quantitative behavioral test for chemonociception (Szolcsányi and Jancsó-Gábor, 1975, 1976). In these experiments the compound was instilled into the right eye of the rats 10 times at 3 min intervals, and the number of protective wipings were counted after each instillation (Fig. 4A). Two hours later the nondesensitizing vanilloid zingerone (1%), the pungent ketone of ginger, was instilled into the treated eye 10 times at 1 min intervals (Fig. 4B). The left eye served as control for a similar series of zingerone instillations (control). Responses to four different vanilloids in four representative experiments and the mean percent (n = 4-8) of "no reactions" of the treated eye (black columns) are seen in the figure. It was remarkable that although some compounds were highly effective in producing nociception, they did not induce "desensitization." Thus, different structural requirements were needed for excitation and for sensory blockade, and the analgesic effect was not due to excessive stimulation of the sensory nerve endings. For example, the homovanilloiloctylester (third row) elicited the most pronounced protective responses without desensitization, while the upper two n-octanoyl-vanilloylamides were highly effective for sensory blockage without causing local anesthesia. In the case of *n*-homovanilloyl-dodecylamide the ratio of concentrations, which elicited a defined level of nociception and desensitization, was 1:1, while for the *n*-heptoyl-vanillylamide this ratio for desensitization was as small as 1:1400. Schematic representation of the hypothetical capsaicin receptor was presented indicating the pharmacophores for pungency and analgesic effects (Szolcsányi and Jancsó-Gábor, 1975, 1976; Szállási and Blumberg, 1999).



FIGURE 4 Relationship between pain-producing and desensitizing activity of a series of capsaicin analogs showing alteration at the site of acylamide linkage only. R, 4-hydroxy, 3-methoxybenzyl group. The vertical lines represent the number of protective wiping movements in response to different compounds instilled into the eye of rats at 3 min intervals (a), and to a 1% solution of zingerone instilled 10 times at 1 min intervals into the pretreated (b) and into the control eye 2 h after the pretreatment with the respective compound. Scale corresponds to 10 wipings. The black columns on the right side of the figure show the percentage of "no reactions" to the compound itself (a) or to zingerone (b) of the treated eye (means of 4-8 experiments). Reprinted from Szolcsányi and Jancsó-Gábor (1976).

The capsaicin receptor concept was not supported for a decade and it was assumed that "the compound presumably affects membrane fluidity and/or ion permeability of the plasma membrane" (Buck and Burks, 1986). From the late 1980s, thorough medicinal chemistry for drug development revealed several structure–activity relationships, which led to the discovery of the first capsaicin receptor antagonist, capsazepine (Bevan *et al.*, 1992). Furthermore, Peter Blumberg and Árpád Szállási identified resiniferatoxin (RTX) as an "ultrapotent" capsaicin analog, which is the irritant principle of a cactus-like plant *Euphorbia resinifera*. The structural moiety of 3-methoxy-4 hydroxy benzyl group or "vanilloyl" domain as they called it was a common structural feature of these two lead molecules. The extremely high potency of

RTX made this compound suitable to elaborate a binding assay (Szállási and Blumberg, 1990), which provided further strong evidence for the existence of a capsaicin receptor in the nociceptive membrane.

Structure-activity relationships in the RTX series of compounds were in several aspects different from those described for the capsaicin analogs (Walpole and Wrigglesworth, 1993; Walpole et al., 1996). Hence a novel concept for different recognition sites for capsaicin and RTX on the TRPV1 capsaicin receptor/ion channel was put forward (Szolcsányi, 2002). It has been stressed that in the case of capsaicin-type vanilloids the 4-OH substitution in the vanilloid structure (V) and its defined position from the NHCO or COO linker region (L) are crucial for gating of the TRPV1 ion channel. The 4-OH substitution and in fact the vanilloid structure itself in RTX, the "ultrapotent" "vanilloid" TRPV1 agonist (Szállási and Blumberg, 1999), is not required for gating of TRPV1. Instead, in the structure of RTX, the presence of polar substitutions of the 5-membered ring (D) of the daphnane diterpene is critical structural requirement for agonism at the TRPV1 receptor. According to this model significance of apolar structures (A) of the agonists and hydrophobic forces to the plasma membrane lipid bilayer (lipid raft) were stressed in binding to and gating of the ion channel. The differences in binding and Ca²⁺ response of RTX and capsaicin were at first interpreted as the existence of distinct R-type and C-type vanilloid receptors (Ács et al., 1997; Szállási et al., 1999). According to the above novel concept (Szolcsányi, 2002), it is explained by two partially different binding sites for capsaicinoids and RTX on the same TRPV1 protein/ion channel. Hence the molecular interaction of the DLA (V) part of the RTX molecule and VL (A) domains for the capsaicin molecule were proposed to play the pivotal role in binding to the TRPV1 protein/lipid raft.

These potential differences in binding sites between capsaicin and RTX to the same ion channel provide an explanation for the paradoxical finding that, although the EC₅₀ for the Ca²⁺ response evoked by capsaicin on rTRPV1- and hTRPV1-transfected cell lines is within the range of 9–74 nM, its K_i value for replacement of ³H-RTX from the receptor is about two orders of magnitude higher (1700–4000 nM). In striking contrast, the K_d value of RTX (0.084–0.13 nM) is lower than its EC₅₀ value (1.4–6.5 nM) on the same cell lines. A further difference between the actions of the two vanilloids is that on cultured dorsal root ganglion (DRG) cell membranes the EC₅₀ for the Ca²⁺ response induced by capsaicin is higher (200–340 nM) than that for its effect on TRPV1-transfected cell lines, while the EC₅₀ of RTX is similar in the two *in vitro* systems (1–2.5 nM). Similar difference in the K_d values of RTX for binding to TRPV1-transfected cells and to cultured DRG neural membranes was not observed (Szolcsányi, 2002; Helyes *et al.*, 2003).

C. Mechanism of Sensory Blockade Induced by TRPV1 Agonists

1. Ultrastructural Changes

The first evidence that capsaicin has a selective long-lasting effect on the cell bodies of B-type small sensory neurons of the dorsal root (Joó et al., 1969) and trigeminal ganglia (Szolcsányi et al., 1975) and on small-type neurons of the preoptic area (Szolcsányi et al., 1971) was obtained by ultrastructural studies. Pronounced mitochondrial swelling that was present several weeks or months after the systemic capsaicin pretreatment was not accompanied by cell death or alterations of the nuclei, endoplasmic reticulum, or Golgi apparatus of the affected neurons. Similar selective mitochondrial damage of the small dark sensory neurons was described after systemic treatment of adult rats with RTX (Szállási et al., 1989; Szolcsányi et al., 1990), or after neonatal pretreatments with capsaicin (Szöke et al., 2002a) (Fig. 5C), or with the TRPV1 agonist endocannabinoid anandamide, which does not have a vanilloid structure (Szöke et al., 2002b). Several hours after five times instillation of a 1% capsaicin solution into the eve of rats in some corneal nerve terminal varicosities partly (>1 µm) or completely uncovered by Schwann cells, swollen mitochondria with disorganized cristae were observed (Fig. 5B). Note the intact mitochondria in the cornea of the solvent-treated side (Fig. 5A) and on the Schwann cell process neighboring the swollen nerve terminal in the capsaicin-treated eye (Fig. 5B). In nerve terminals having swollen mitochondria, the number of microvesicles was markedly diminished (3.6 \pm 3.2 per square micrometer, n = 7) compared to that of the nerve terminals in the control cornea (48 \pm 28.6 per square micrometer, n = 11) (Szolcsányi et al., 1975). A more pronounced damage with loss of nociceptive epidermal nerve terminals after topical application of a capsaicin cream (0.075% w/w) was observed in the human skin (Nolano et al., 1999) and was described in the skin and cornea of rats after systemic capsaicin treatment (Chung et al., 1990; Szolcsányi, 1991, 1996a).

Pretreatment of neonatal rats with a 50 mg/kg s.c. dose of capsaicin induced an irreversible loss of B-type neurons and some A-type neurons of the spinal ganglia accompanied by a substantial loss of unmyelinated and some myelinated afferent fibers as detected 3–6 month later (Jancsó *et al.*, 1977; Lawson, 1987; Szolcsányi *et al.*, 1994; Szöke *et al.*, 2002a). According to the original description necrotic-like cell death of B-type sensory neurons occurred within 30 min and it was claimed that the functional blockade induced by capsaicin *in vivo* is due to this "highly selective" acute neurotoxic effect of capsaicin on chemonociceptive neurons (Jancsó *et al.*, 1977; Jancsó and Király, 1981). However, no quantitative morphometric data supported these observations and conclusions. Therefore these early data and the popular neurotoxin concept for the *in vivo* actions of capsaicin



FIGURE 5 Panel A shows sensory nerve ending in the cornea of a control rat. M: mitochondria; mv: microvesicles; coll: collagen fibers. ×47000. Panel B shows sensory nerve ending in the capsaicin-treated cornea of a rat. A 1% solution of capsaicin was instilled 46, 26, 22, 18, and 5 h before euthanization. M_1 : swollen mitochondrion in the nerve terminal; M: mitochondrion in the Schwann cell process. ×47000. Panel C shows electron micrograph of B-type cells from a 6-week-old capsaicin-treated animal. Six weeks after the capsaicin treatment many neurons displayed a cytoplasm with almost no intact mitochondria. The mitochondria are swollen and appear to be empty, containing no internal cristae. The cell nucleus shows no sign of damage, the nuclear chromatin is evenly distributed and the nucleolus shows an intact inner structure. Scale bar = 2.5 μ m. Panel D shows: (a) The number of A- and B-type neurons of trigeminal ganglia at the fifth day after neonatal capsaicin treatment (50 mg/kg s.c., n = 6), and of 1-week-old control trigeminal ganglia (n = 4). Cell number of the capsaicin-treated rats was

needed revision. It turned out that in neonatal rats similarly as in the adults, capsaicin pretreatment induced a long-lasting selective mitochondrial damage of the B-type neurons (Fig. 5C) and there was no significant loss of sensory neurons within 5 days (Fig. 5D). Nineteen days after the treatment a selective loss of B-type neurons appeared, but it was prevented in rats which were treated with nerve growth factor (NGF) from 1 day after the capsaicin injection had been completed (Fig. 5D). Hence, it has been suggested that in both neonatal and adult rats, systemic application of capsaicin in high doses induces a selective pronounced mitochondrial swelling in the cell body and destruction of their terminal nerve processes. After capsaicin treatment, the impaired uptake of NGF results in cell death in neonatal rats but not in the adults (Szöke et al., 2002a,b). Furthermore, it should be kept in mind that in adult rats pretreated at a neonatal age, there is an indiscriminate loss of Cafferents in contrast to the selective reduction of C-polymodal nociceptors, which occurred after adult treatment (Szolcsányi, 1993; Szolcsányi et al., 1994; Szöke et al., 2002a). This observation points to phenotypic switches of C-afferents during ontogenic development of rats, which lost a substantial portion of their C-polymodal nociceptive neurons.

2. Functional Desensitization of C-Polymodal Nociceptors

Under *in vivo* conditions the primary site of action of TRPV1 agonists is at the nociceptive nerve terminal and not on its axonal trunk. Close-arterial infusion of capsaicin into the femoral artery of rats does not evoke action potentials if the nerve trunk is perfused, while similar doses of capsaicin excited the peripheral and central terminals of primary sensory neurons (Pethö and Szolcsányi, 1996). In a single unit study on rabbits, close-arterial injection of capsaicin into the main artery of the ear desensitized single Cpolymodal nociceptors against the actions of noxious heat and mechanical stimuli similarly to capsaicin, bradykinin, or xylene (Szolcsányi, 1987). It has been suggested that the transducer mechanism of C-polymodal nociceptors is desensitized since the ineffectiveness or decreased responsiveness to different stimuli ensued in a nonparallel fashion and conduction velocity of the axon remained unchanged (Szolcsányi, 1987, 1993). Evidence for an action of capsaicin on the transducer portion and not on the terminal axon is provided also by the lack of an inhibitory effect of local anesthetics or

not significantly different to that counted in the trigeminal ganglia from control rats. (b) The number of A- and B-type neurons of trigeminal ganglia 19 days after neonatal capsaicin treatment (50 mg/kg s.c., n = 4) and capsaicin+NGF treatment (50 mg/kg s.c. and 10 × 100 µg/kg s.c., n = 5). Cell number of 3-week-old control trigeminal ganglia is also presented (n = 4). Results are shown as means ' SEM, *p < 0.05 indicates a significant difference. Panel A and B are reprinted from Szolcsányi *et al.* (1975), and C and D from Szöke *et al.* (2002a).

tetrodotoxin (TTX) on the capsaicin-induced release of sensory neuropeptides in an isolated tissue preparation. These pretreatments prevented completely the effect of electrical nerve stimulation (Németh *et al.*, 2003). In rats after systemic capsaicin pretreatment, the threshold of C-polymodal nociceptors to capsaicin and mechanical stimuli is increased (Szolcsányi *et al.*, 1988; Szolcsányi, 1993).

Hence binding of vanilloid (capsaicin, RTX) or nonvanilloid agonists (anandamide, *N*-oleoyldopamine) to the TRPV1 ion channel alters the function of the nociceptive neuron *in vivo* at four stages (Szolcsányi, 1993, 2004; Szolcsányi *et al.*, 1994).

The first stage of agonism is gating of the TRPV1 cation channel that results in depolarization followed by propagation of impulses and Ca^{2+} -influx, which induces release of sensory neuropeptides including tacykinins, calcitonin gene-related peptide (CGRP), and somatostatin. The second stage is the sensory neuron blockade characterized by a decreased function of the nerve terminals, which manifests itself as an attenuation of their responsive-ness to physical and chemical stimuli, and as a decreased sensory-efferent function due to a diminished neuropeptide release. At this stage ultrastructural changes are present without degeneration. The third stage is characterized by degeneration of the terminal axon and mitochondrial damage in the somata of nociceptive and preoptic neurons. The fourth stage is cell death which ensues *in vivo* in neonatal animals when the NGF uptake is inhibited in damaged nerve terminals or when hypoxic condition due to respiratory distress caused by the capsaicin treatment is combined with long-lasting TRPV1 agonism.

D. Discovery of the Capsaicin-Gated Cation Channel

The remarkably selective action of capsaicin on a subpopulation of sensory neurons activated also by bradykinin has opened up new means to study under *in vitro* conditions ion channels sensitive to capsaicin (Baccaglini and Hogan, 1983). Measurements of ion fluxes in neurons of dorsal root ganglia in cell culture provided the first evidence for the existence of a capsaicingated cation channel (Wood *et al.*, 1988). Furthermore, it has been shown that capsaicin or RTX activates single channel currents in isolated membrane patches from capsaicin-sensitive DRG neurons (Bevan and Szolcsányi, 1990; Oh *et al.*, 1996). Direct validation of these data was achieved after cloning of the TRPV1/VR1 capsaicin-gated cation channel (Caterina *et al.*, 1997). Cloning strategy to identify similar temperaturegated ion channels that are gated by noxious thermal stimuli led to the discovery of the TRPV2/VRL1 ion channel (Caterina *et al.*, 1999), which is gated by higher noxious temperatures (threshold about 53° C) and the TRPV3 cation channel that is gated by increasing temperature both in nonnoxious and noxious range (Smith *et al.*, 2002). Out of the two cation channels responding to cooling the channel TRPA1/ANKTM1 is gated by noxious cold (<18°C) while TRPM8 responds to menthol and cooling below 28°C (for more details see Chapter 9). The former channel seems to subserve nociceptive transducer functions while the TRPM8 is expressed in cold sensor afferents. Further members of the TRPV family are apparently not involved in nociceptive functions. Surprisingly, unlike TRPV1, none of these cation channels responds to vanilloids.

III. VOLTAGE-GATED NA⁺ CHANNELS

Voltage-gated Na⁺ channels mediate the initial depolarization responsible for the rising phase or upstroke of the action potential in excitable tissues including neurons, skeletal, and cardiac muscle. They consist of an α subunit sufficient for functional channel expression and associated auxiliary β subunits modifying kinetics and voltage-dependence of gating. The α subunit is composed of four homologous domains (I-IV), each containing six transmembrane α helices (S1–S6) and a pore loop located between S5 and S6. These channels are blocked by local anesthetics, class I antiarrhythmic drugs, and certain antiepileptic drugs. It turned out relatively early that Na⁺ channels were not pharmacologically homogenous because they exhibit remarkably different sensitivity to the blocking action of TTX, a higly potent toxin from a Pacific fish, the pufferfish—Na⁺ channels that are blocked by low nanomolar concentrations are called TTX-sensitive (TTX-S) whereas those requiring more than one micromolar are considered TTX-resistant (TTX-R). Both TTX-S and TTX-R Na⁺ currents have been described in neuronal, denervated skeletal muscle and cardiac muscle preparations. Several TTX-S and TTX-R Na⁺ channel subtypes have been identified by molecular cloning and they are characterized by different tissue distributions, electrophysiological features, and pharmacological modulation (see Table I for a short summary).

A. Summary of Early Observations on Voltage-Gated Na⁺ Currents in Primary Afferent Neurons

A number of studies in the late seventies and in the eighties have indicated that at least two kinetically and pharmacologically distinct Na^+ currents can be recorded from somata of primary sensory neurons (Caffrey *et al.*, 1992;

Official name	Other name(s)	TTX-sensitivity	Localization
Na _v 1.1	Brain type I	Sensitive	CNS, DRG, motor neurons
Na _v 1.2	Brain type II	Sensitive	CNS
Na _v 1.3	Brain type III	Sensitive	Adult CNS, embryonic DRG
Na _v 1.4	SkM1	Sensitive	Skeletal muscle
Na _v 1.5	H1	Resistant	Heart, embryonic DRG
Na _v 1.6	PN4	Sensitive	DRG, CNS
Na _v 1.7	PN1, hNE, NaS	Sensitive	DRG, CNS
Na _v 1.8	SNS, PN3	Resistant	DRG
Na _v 1.9	NaN, SNS2, PN5	Resistant	DRG

 TABLE I

 Some Features of the Voltage-Gated Na⁺ Channel α Subunits

CNS, central nervous system; DRG, dorsal root ganglion; TTX, tetrodotoxin.

Rush *et al.*, 1998; Scholz *et al.*, 1998). One of them is a TTX-S current that is expressed either alone or with a TTX-R current. The TTX-S current is characterized by a low threshold for activation, rapid activation, and inactivation as well as typically a slow recovery from inactivation (slow repriming). Neuronal TTX-R currents were reported in DRG neurons of the rat, mouse, and human as well as in nodose ganglion neurons of the rat. The predominant TTX-R current in the majority of DRG neurons has a higher threshold for activation, together with a slower activation and inactivation kinetics compared to the TTX-S current. In some studies and in a small number of DRG neurons, a quite distinct TTX-R current with fast inactivation kinetics was also observed and denoted as TTX-R3.

A study has compared the contribution of TTX-S and TTX-R Na⁺ channels to the generation of the action potential in the cell body of small (predominantly nociceptive) DRG neurons (Blair and Bean, 2002). TTX-R channels were shown to play the dominant role in carrying the charge at all stages of the action potential from threshold to the falling phase. The slow and incomplete inactivation of TTX-R channels during the action potential can explain the long duration of action potentials in nociceptive primary sensory neurons. It has been shown that slow inactivation of the TTX-R current plays an important role in adaptation of action potential firing in small DRG neurons and may contribute to cross-desensitization between chemical and electrical stimuli (Blair and Bean, 2003).

While unequivocal evidence indicates that in somata of primary afferent neurons TTX-R action potentials can be recorded, a number of studies

revealed that action potential conduction in both myelinated and unmyelinated sensory axons of peripheral nerve trunks were sensitive to blockade by TTX (Strassman and Raymond, 1999). This is most likely due to a lower level of TTX-R channel expression along the axons compared to somata of primary sensory neurons. Some studies, however, provided opposite results. In an in vitro spinal cord-DRG preparation, TTX failed to inhibit action potential propagation in slowly conducting (but not in fast-conducting) afferents; and high potassium applied to DRG, dorsal root, or peripheral process activated a primary afferent input, which was Na⁺-dependent but TTX-R (Jeftinija, 1994). Similarly, TTX-R action potentials were revealed in biopsy material of the human sural nerve (Quasthoff et al., 1995). The results of two studies support the presence of TTX-R Na⁺ channels not only in peripheral axons of sensory nerves but also in the nerve endings, that is, the generator region of nerve terminals. Electrophysiological evidence for TTX-R Na⁺ currents in slowly conducting A δ and C sensory fibers in the dura mater was provided (Strassmann and Raymond, 1999). Considering the experimental arrangement of this study, the results are compatible with the hypothesis that the TTX-R Na⁺ channels revealed functionally were preferentially localized in the nerve endings and preterminal axonal branches of dural C-fibers. Evidence supporting the involvement of TTX-R channels in generating nerve impulses in the generator region of peripheral terminals of nociceptive C-fibers has been provided in the cornea of the guinea pig (Brock et al., 1998).

B. Identification and Characterization of Na⁺ Channels in Primary Afferent Neurons

1. TTX-S Na⁺ Channels

The voltage-gated Na⁺ channels responsible for TTX-S and TTX-R currents in primary sensory neurons remained elusive until the middle of the nineties while those in brain, skeletal, and cardiac muscle had already previously been identified. In 1993, a novel subtype of Na⁺ channels was identified by molecular cloning in PC12 cells whose messenger RNA (mRNA) was found in dorsal root and sympathetic ganglionic neurons as well, therefore, this channel was designated as peripheral nerve type 1 (PN1), now called Na_v1.7 (D'Arcangelo *et al.*, 1993). Later a new isoform of Na⁺ channels was cloned from human medullary thyroid carcinoma cells (Klugbauer *et al.*, 1995). This channel proved to be TTX-S and was shown to be expressed in various neuroendocrine cells including the thyroid and adrenal glands, and it was termed human neuroendocrine (hNE). The same Na⁺ channel α subunit was also cloned from rabbit Schwann cells and accordingly

named NaS (Belcher et al., 1995). Later PN1 was cloned from rat and human DRGs, and it turned out that hNE was the human ortholog of both PN1 and NaS (Sangameswaran et al., 1997; Toledo-Aral et al., 1997). Na_v1.7 (PN1/hNE/NaS) was found in many different peripheral tissues in addition to small and large DRG neurons. The intracellular localization of Nav1.7 in DRG neurons is unique because it is found not only in somata and axons but also in peripheral terminals where the highest amounts were detected (Toledo-Aral et al., 1997). In another study its mRNA was found in most cells in all size classes of DRG neurons (Black et al., 1996). The predominant TTX-S Na⁺ current in small DRG neurons from adult rats has slow repriming kinetics (Cummins and Waxman, 1997; Rush et al., 1998). Na_v1.7 expressed in HEK293 cells evoked Na⁺ currents that were characterized by slow closed-state inactivation and slow recovery from inactivation, similar to TTX-S currents recorded from small DRG neurons (Cummins et al., 1998). These findings suggest that $Na_v 1.7$ is the major Na^+ channel underlying TTX-S currents in nociceptive primary afferent neurons. Owing to slow closed-state inactivation, Nav1.7 channels can respond to small, slow depolarizations close to resting potential and produce depolarizing persistent currents that can amplify inputs such as generator potentials (Cummins et al., 1998; see also Waxman et al., 2000). Such a function for Nav1.7 channels is further supported by their revealed preferential localization in axon terminals. It is worth mentioning that a TTX-R Na⁺ channel with similar functional characteristics and localization is also found in small-type primary sensory neurons (see Na_v1.9 in the following section). In a study, Na_v1.7 protein was found in all C, 90% of A δ and 40% of A α/β units, including both nociceptive and low-threshold mechanoceptive units in the guinea pig DRG neurons, with a higher proportion of the nociceptive ones (Djouhri et al., 2003).

Another TTX-S channel, $Na_v 1.6$, has been localized in nodes of Ranvier in myelinated fibers along both motor and sensory axons in the peripheral and central nervous system (Caldwell *et al.*, 2000). Subsequently, this channel has been revealed in unmyelineted C-afferents as well from terminal receptor fields in the skin to the dorsal root entry zone in the spinal cord (Black *et al.*, 2002). It was also found in the nerve endings of corneal C-fibers. By examining mice lacking the Na_v1.6 gene, it was shown that Na_v1.6 contributed to action potential conduction in unmyelinated fibers indicating that not only saltatory but also continuous conduction involves Na_v1.6 Na⁺ channels.

A detailed comparative analysis has provided evidence that mRNAs of TTX-S Na⁺ channel α subunits are differentially expressed in the small, medium, and large DRG neurons from adult rats while β 1 and β 2 subunit mRNAs exhibit similar expression patterns (Black *et al.*, 1996). Na_v1.1 (brain type I) is expressed at higher levels in large than small neurons,

 $Na_v 1.2$ (brain type II) is variably expressed, with most cells lacking or exhibiting only low levels. $Na_v 1.3$ (brain type III) is found only at very low levels or not at all. By contrast, $Na_v 1.3$ mRNA is expressed considerably during embryonic development (Waxman *et al.*, 1994). $Na_v 1.7$ mRNA was detected at moderate or high levels in most cells in all size classes. It was also shown that the expression pattern in dissociated adult DRG neurons at 1 day *in vitro* was similar to that exhibited by adult DRG neurons *in situ*.

2. TTX-R Na⁺ Channels

Akopian et al. (1996) reported cloning of a TTX-R Na⁺ channel from the rat whose mRNA was expressed only by small diameter sensory neurons in dorsal root and trigeminal ganglia and which was accordingly termed sensory neuron-specific (SNS), now called Na, 1.8. The electrophysiological properties of SNS expressed in oocytes were similar to those of the typical TTX-R currents in small DRG neurons including high threshold for activation and relatively depolarized voltage dependence of steady-state inactivation. Two months later, another paper appeared reporting the cloning of an almost identical rat protein, termed peripheral nerve type 3 (PN3), with similar preferential localization in small DRG neurons and functional characteristics including slow inactivation as well (Sangameswaran et al., 1996). According to a subsequent study, Nav1.8 (SNS/PN3) mRNA is found in all size classes of DRG neurons but greater levels can be detected in small and medium neurons than in large ones (Black et al., 1996). Subsequently, the mouse and human cognates of Na_v1.8 were also cloned (Souslova *et al.*, 1997; Rabert et al., 1998). Nuclear injection of Nav1.8 cDNA into sensory neurons isolated from Nav1.8 null mutant mice evoked expression of a TTX-R current similar to that observed in DRG neurons (Akopian et al., 1999). Another study on Na_v1.8 knockout mice revealed that 80-90% of the inward current that flows during the rising phase of the action potential in small DRG neurons was mediated by Nav1.8 showing the importance of this TTX-R Na⁺ channel in electrogenesis of nociceptive primary sensory neurons (Renganathan et al., 2001). Nav1.8 also displays a rapid recovery from inactivation, which, together with the relatively depolarized voltage dependence of steady-state inactivation, might allow Na_v1.8-expressing neurons to sustain repetitive firing at depolarized membrane potentials when other Na⁺ channels are inactivated. Nav1.8, unlike other Na⁺ channels, is poorly expressed in cell lines even in the presence of accessory β subunits. A regulatory protein, annexin II light chain (p11) was identified, which can bind to Nav1.8 and thereby promote its translocation to the plasma membrane, resulting in functional channels (Okuse et al., 2002). Knockdown of p11 expression (by an antisense oligodeoxynucleotide) inhibited Nav1.8 Na⁺ currents in sensory neurons.

In 1998, a second subtype of TTX-R voltage-gated Na⁺ channels, initially termed NaN (novel and nociceptive, now called Na, 1.9), expressed preferentially in small primary sensory neurons of dorsal root and trigeminal ganglia was cloned from the rat and 1 year later from the mouse (Dib-Hajj et al., 1998a, 1999a). Heterologous expression of this protein, termed also SNS2, in HEK293T cells resulted in TTX-R Na⁺ currents (Tate et al., 1998). Surprisingly, no TTX-R currents were recorded in DRG neurons in the first study on mice lacking the gene for $Na_v 1.8$ (Akopian *et al.*, 1999). In a subsequent study, however, a unique TTX-R Na⁺ current was observed in small DRG neurons of both Na_v1.8 null mutant and wild-type mice, which was attributed to Nav1.9 (Cummins et al., 1999). This current was characterized by a lower threshold (near -70 mV) for activation, slower rate of activation, and faster inactivation rate compared to $Na_v 1.8$ and was persistent at negative potentials close to resting potential (Dib-Hajj et al., 2002). Subsequently, the Na_v1.9 channel was also cloned from human DRG and two different types of TTX-R Na⁺ currents were described in human primary sensory neurons: one resembling the current specific for Na_v1.8 and another most likely mediated by Na_v1.9 (Dib-Hajj *et al.*, 1999b). A study employing computer simulation suggested that the persistent TTX-R current mediated by Na_v1.9 channels was not involved in the generation of action potentials owing to its slow activation kinetics, rather it contributed a depolarizing influence to resting membrane potential and could amplify subthreshold depolarizations (Herzog et al., 2001). Evidence has been provided that the Nav1.9 protein is preferentially localized not only in somata but also along axons of IB₄positive DRG neurons connected to unmyelinated fibers in the sciatic nerve, in clusters at the nodes of Ranvier of thinly myelinated fibers in the sciatic nerve as well as in axon terminals of the cornea (Fjell et al., 2000). In a subsequent study, both Nav1.8 and Nav1.9 were found along unmyelinated axons and at their terminals within superficial epithelial layers of the cornea (Black and Waxman, 2002). These findings are in full accord with previous functional studies revealing TTX-R currents in nerve terminals of corneal and dural nociceptive afferents (Brock et al., 1998; Strassman and Raymond, 1999). They also support the hypothesis that TTX-R Na⁺ channels are found not only at the regenerative region of nociceptors but also within the generator region where they might facilitate generator potentials. The Nav1.9 protein was found exclusively in nociceptive (various types of C- and A-fibers) but not in the low-threshold mechanoreceptive DRG neurons as determined by direct assessment of their sensory responsiveness (Fang et al., 2002). The intensity of Na_v1.9-like immunoreactivity was negatively correlated with soma size and conduction velocity of C-fiber units whereas a positive correlation was revealed with action potential duration, especially with rising time. Analogously to the interaction of Na_v1.8 with annexin light chain (see earlier), contactin was shown to bind directly to $Na_v 1.9$ and facilitate its targeting to cell membrane of DRG neurons (Liu *et al.*, 2001).

A comparative study has revealed that $Na_v 1.9$ is found exclusively in small DRG neurons connected to unmyelinated C-fibers, whereas $Na_v 1.8$ is localized in both C- and myelinated A-fiber DRG neurons (Amaya *et al.*, 2000). About half of C-fiber DRG neurons express either $Na_v 1.8$ or $Na_v 1.9$, and in most cells, the two channels are colocalized. $Na_v 1.8$ and $Na_v 1.9$ are found both in NGF-responsive and glial cell-derived neurotrophic factor (GDNF)-responsive C-fiber neurons, most of which express the capsaicin receptor TRPV1 (Amaya *et al.*, 2000). Consistent with this, significantly higher levels of TTX-R currents were found in capsaicin-sensitive DRG neurons than in the capsaicin-insensitive ones while no difference in the levels of TTX-S current was revealed (Arbuckle and Docherty, 1995). It is worth mentioning that expression of both $Na_v 1.8$ and $Na_v 1.9$ increases with embryonic age (Benn *et al.*, 2001) indicating that the developmental regulation of Na^+ channel expression is a complex process affecting several Na^+ channel subtypes (see also $Na_v 1.3$ given earlier and $Na_v 1.5$ in the following paragraph).

In addition to the Na_v1.8-mediated slow-inactivating and the Na_v1.9mediated persistent current, a fast-inactivating TTX-R current has also been revealed in a very small number of small and medium-sized DRG neurons and was called TTX-R3 current (Rush *et al.*, 1998; Scholz *et al.*, 1998). Of the TTX-R Na⁺ channels, the cardiac type, Na_v1.5 (rat heart type 1, rH1), produces fast-inactivating TTX-R currents, but this channel was not found in DRG neurons from adult rats (Black *et al.*, 1996). A study has shown that both fast-inactivating TTX-R currents and Na_v1.5 mRNA are present in a large number of embryonic DRG neurons with a declining tendency as embryonic development proceeds (Renganathan *et al.*, 2002). This study found fast-inactivating TTX-R currents only in 3% of adult small DRG neurons. The voltage-dependence, kinetics, TTX, and cadmium sensitivity of these currents are similar to those described for the cardiac Na⁺ channel suggesting that Na_v1.5 mediates the fast-inactivating TTX-R3 current in embryonic (and very rarely in adult) DRG neurons.

C. Alterations in Expression and Function of Na⁺ Channels in DRG Neurons Under Neuropathic Conditions

One of the first experimental findings was that $Na_v 1.3 \text{ mRNA}$, which is expressed considerably during embryonic development but not in adult animals, is upregulated in axotomized DRG neurons of adult rats indicating a reversion to an embryonic pattern of Na^+ channel expression by nerve injury (Waxman *et al.*, 1994). Axotomy also leads to a downregulation of Nav1.8 mRNA in adult DRG neurons (Dib-Hajj et al., 1996). Nav1.8 mRNA downregulation following axotomy was also reveled in small-sized neurons of the trigeminal ganglia (Bongenhielm et al., 2000). It was also demonstrated in large cutaneous afferent neurons that following axotomy rapidly inactivating, TTX-S currents were enhanced whereas the slowly inactivating, TTX-R currents were attenuated (Rizzo et al., 1995). Downregulation of TTX-R currents accompanied by a decrease in Nav1.8 mRNA levels was revealed in axotomized small spinal DRG neurons as well (Cummins and Waxman, 1997). In these neurons the TTX-S current density, however, was not altered by axotomy, but upregulation of a rapidly repriming TTX-S current and Nav1.3 mRNA was observed suggesting downregulation of a slowly recovering TTX-S current, most probably mediated by $Na_v 1.7$. Subsequently, immunocytochemical evidence has been provided that following transection of the sciatic nerve the Na_v1.3 protein is upregulated parallel to the emergence of the rapidly repriming TTX-S current, and that Na_v1.3 is localized in not only the somata but also the axons of axotomized primary afferent neurons (Black et al., 1999). A further finding was that dorsal rhizotomy (transection of the central axonal projections in the dorsal roots) failed to induce such changes. TTX applied to the DRG of the ligated spinal nerve reduced ectopic discharges in injured sensory neurons and diminished concomitant mechanical allodynia. In accord with this, L4/L5 spinal nerve ligation led to a significant upregulation of $Na_v 1.3$ with a concomitant downregulation of Nav1.1 and Nav1.2 TTX-S Na⁺ channels, preferentially in medium and large neurons of the affected DRG (Kim et al., 2001).

Fully in accord with the previous results, both Na_v1.8 and Na_v1.9 proteins are downregulated in small DRG neurons following sciatic nerve transection together with a decrease in both slowly inactivating and persistent TTX-R currents; dorsal rhizotomy, however, failed to evoke changes in either channel expression or membrane currents indicating that axotomy per se is not responsible for these changes (Sleeper *et al.*, 2000). The most likely explanation is that peripheral axotomy causes a deprivation of tissue-derived neurotrophic factors in the proximal parts of the injured sensory neurons by blocking their centripetal axonal transport from the peripheral terminal (see also Section III.D).

Expression of Na_v1.8 and/or Na_v1.9 mRNA/protein in DRG neurons was shown to diminish in three other models of neuropathic pain: L5/L6 tight spinal nerve ligation, chronic constriction injury of the sciatic nerve, and streptozotocin-induced diabetes (Okuse *et al.*, 1997; Craner *et al.*, 2002; Decosterd *et al.*, 2002). In the latter model mRNAs for Na_v1.3, Na_v1.6, and Na_v1.9 were upregulated whereas those for Na_v1.1 and Na_v1.7 were unaffected (Craner *et al.*, 2002). Furthermore, in streptozotocin-induced diabetes the intensity of TTX-R current of DRG neurons was increased and channels were activated at more negative potentials indicating an enhanced excitability (Hirade *et al.*, 1999). Spinal cord transection results in urinary bladder hyperactivity, which is associated with a diminishment of TTX-R currents, strong downregulation of Na_v1.8, and slight upregulation of Na_v1.9 in bladder afferents (Black *et al.*, 2003). The studies mentioned above have provided ample evidence that various forms of peripheral nerve damage induce a shift in Na⁺ channel expression in the injured primary afferent neurons in that TTX-R channels (Na_v1.8 and/or Na_v1.9) are downregulated and TTX-S channels (Na_v1.3) are upregulated (Lai *et al.*, 2004).

On the other hand, however, both TTX-R and TTX-S currents of DRG neurons were upregulated coinciding with their hyperexcitability in a study (Abdulla and Smith, 2002). Other studies have revealed that vagotomy leads to a decrease in excitability of neurons in the nodose ganglion together with a decrease in TTX-R current and increase in TTX-S current (Lancaster and Weinreich, 2001; Lancaster *et al.*, 2001). These results show that axotomy-induced functional changes in primary sensory neurons are not uniform and may depend on cell type. In a study, 10 days post-axotomy the TTX-R current decreased and the TTX-S current increased in injured DRG neurons but excitability was unaltered. Four weeks post-axotomy, injured DRG neurons were hyperexcitable, their TTX-R current was still decreased but the TTX-S current returned to control level (Flake *et al.*, 2004). It indicates that a change in Na⁺ current does not necessarily result in a change in excitability, and reduction of the TTX-R current, rather than increase in TTX-S current in injured DRG neurons seems to be associated with hyperexcitability.

A specific antisense oligodeoxynucleotide to Na_v1.8 applied intrathecally in rats evoked a decrease in both Nav1.8 expression and slow-inactivating TTX-R Na⁺ current of DRG neurons and concomitantly eliminated tactile allodynia and heat hyperalgesia induced by L5/L6 spinal nerve ligation (Lai et al., 2002; Gold et al., 2003). In Nav1.8 null mutant mice spontaneous activity of units from neuromas formed by transection of the saphenous nerve was drastically reduced compared to wild-type littermates indicating the essential role of Na_v1.8 in this model of neuropathic pain (Roza *et al.*, 2003). In contrast, a previous study reported no change in neuropathic pain behaviors following partial nerve injury in mice lacking the Nav1.8 gene (Kerr et al., 2001). It should be emphasized, however, that a compensatory increase in TTX-S currents has been observed in Nav1.8 knockout mice (Akopian *et al.*, 1999), which could mask the effect of lacking $Na_v 1.8$. These studies suggest that $Na_v 1.8$ activation might be involved in the hyperalgesia following peripheral nerve injury. The functional features of this Na⁺ channel, such as the depolarized voltage dependence for, and rapid recovery from, inactivation, suggest that it could cause sustained repetitive firing at depolarized membrane potentials characteristic for damaged neurons. However, strong evidence indicates the downregulation of this Na^+ channel type in various neuropathic conditions (see earlier). This apparent contradiction might be resolved by data obtained in those studies that investigated the level of $Na_v 1.8$ expression not only in somata but also in the axons of sensory neurons and which considered the neighboring, uninjured neurons as well (Lai *et al.*, 2004).

Chronic constriction injury to the rat sciatic nerve resulted in no change in the densities of TTX-S or TTX-R currents and Na, 1.8 mRNA expression in DRG neurons but a redistribution of the Na_v1.8 protein was revealed: Nav1.8 immunolabeling decreased in the somata and increased in the injured peripheral axons at the site of injury suggesting a translocation of the presynthesized channel protein from soma to the periphery; this redistribution was observed following complete sciatic nerve transection as well (Novakovic et al., 1998). Na_v1.8 accumulation in nerve fibers just proximal to the injury was also revealed in human nerves, in vitro (Yiangou et al., 2000). In a human study, destruction of central axons caused by brachial plexus injury resulted in downregulation of both $Na_v 1.8$ and $Na_v 1.9$ proteins in cervical DRG neurons (Coward et al., 2000). In contrast, an upregulation of both channels was observed in peripheral nerve fibers just proximal to the site of injury and in neuromas. Axonal content of these channels was not altered by various neuropathic conditions including nerve inflammation or demyelination. In various neuropathic models including L5/L6 spinal nerve ligation and chronic constriction injury of the sciatic nerve, expression of both Na_v1.8 and -1.9 proteins in the injured DRG neurons was abolished, while in intact DRGs and neighboring uninjured neurons no change in channel expression was revealed (Decosterd et al., 2002). In another study, L5/L6 spinal nerve ligation led to an accumulation of Na_v1.8 protein in uninjured axons of the sciatic nerve originating from the L4 DRG whereas no such effect was observed in cell bodies of L4 DRG neurons (Gold et al., 2003). These results show that Nav1.8 expression is maintained or even increased at axonal sites proximal to injury and in neighboring uninjured sensory neurons, which can explain the pathogenetic role of $Na_v 1.8$ in various neuropathic conditions suggested by the Nav1.8 antisense and knockout studies.

Regarding the functional role of $Na_v 1.9$ downregulation in hyperexcitability observed after nerve injury, a hypothesis was put forward by Cummins and Waxman (1997) (Waxman *et al.*, 1999). Following axotomy, downregulation of $Na_v 1.9$ and the subsequent loss of its persistent depolarizing influence would cause a hyperpolarizing shift in resting potential and remove resting inactivation on TTX-S channels. In other words, a partial deficiency of one type of a TTX-R channel would have a facilitatory effect on the upregulated TTX-S channels. A similar mechanism was revealed in axons of the rat optic nerve (Stys *et al.*, 1993). In a subsequent study, however, an antisense oligodeoxynucleotide to $Na_v 1.9$, albeit reduced $Na_v 1.9$ expression, failed to alter mechanical or heat hyperalgesia induced by spinal nerve ligation (Porreca *et al.*, 1999). At present no convincing evidence exists for the role of $Na_v 1.9$ in neuropathic pain.

Finally, the role of Na^+ channel activation in neuropathic pain is supported by the clinical efficacy of certain Na^+ channel blockers, such as lidocaine, mexiletine, carbamazepine, phenytoin, and lamotrigine, to reduce spontaneous pain and hyperalgesia/allodynia in various neuropathic diseases (Lai *et al.*, 2004).

D. Influence of Neurotrophic Factors on Na⁺ Channel Expression and Na⁺ Currents in DRG Neurons

Nerve growth factor is considered as a trophic factor produced in peripheral tissues, which is necessary for the survival during development and maintenance of the normal phenotype in adult animals of a subpopulation of nociceptive primary afferent neurons expressing the high-affinity receptor TrkA. In cultured DRG neurons NGF can enhance TTX-R current acutely (Zhang *et al.*, 2002). NGF was shown to upregulate Na_v1.8 but not Na_v1.9 channels in isolated small sensory neurons (Black *et al.*, 1997; Fjell *et al.*, 1999a). Experimental gastric ulcer can lead to an upregulation of NGF expression in the gastric wall, and exogenously applied NGF increases TTX-R but not TTX-S current in gastric sensory neurons (Bielefeldt *et al.*, 2003). Brain-derived neurotrophic factor (BDNF), however, failed to alter Na⁺ currents in DRG neurons (Waxman *et al.*, 1999).

Peripheral nerve transection prevents retrograde transport of NGF in primary afferent neurons, and NGF deficiency in the soma may account for the phenotypic changes that appear in DRG neurons following axotomy. NGF was shown to induce Na_v1.7 mRNA in PC12 cells (D'Arcangelo *et al.*, 1993; Toledo-Aral *et al.*, 1995, 1997). In accordance with this, exogenous NGF was able to attenuate alterations of Na⁺ channel mRNA levels characteristic for axotomy, decreasing Na_v1.3 mRNA and increasing Na_v1.8 mRNA expression in dissociated rat small DRG neurons *in vitro* (Black *et al.*, 1997). Furthermore, NGF applied to the proximal stump of the transected sciatic nerve of rats *in vivo*, resulted in an almost complete restoration of diminished Na_v1.8 mRNA expression but only a partial normalization of decreased TTX-R currents in small DRG neurons (Dib-Hajj *et al.*, 1998b). Glial cell-derived neurotrophic factor (GDNF) is important for the maintenance of phenotypic properties in a subpopulation of primary afferent neurons lacking the TrkA receptor for NGF. NGFsensitive and GDNF-sensitive primary afferent neurons can be differentiated by their different ability to bind the plant lectin IB_4 (Stucky and Lewin, 1999). While Na_v1.8 mRNA is expressed both in IB₄-positive and -negative neurons, $Na_v 1.9$ is preferentially expressed in IB₄-positive neurons (Fiell et al., 1999a). Therefore, Nav1.8 may underlie much of the slow TTX-R current observed in IB₄-negative neurons while Na_v1.9 seems responsible for that recorded in IB₄-positive cells. Exogenously applied NGF was shown to restore levels of Nav1.8 mRNA in both IB4-positive and -negative neurons but the level of Nav1.9 mRNA remained unaltered (Dib-Hajj et al., 1998b; Fjell et al., 1999a). In contrast, GDNF treatment restored both Nav1.8 and Na_v1.9 mRNA in IB₄-positive neurons (Fiell et al., 1999b). In vivo NGF deprivation was shown to reduce Na_v1.8 expression and TTX-R Na⁺ currents in IB₄-negative DRG neurons of rats (Fjell et al., 1999b). GDNF was reported to increase mRNA and protein levels of both Nav1.8 and Nav1.9 channels as well as to enhance both the slow-inactivating and persistent TTX-R currents in axotomized DRG neurons both in vitro and in vivo (Cummins et al., 2000). Moreover, in rats with L5 spinal nerve ligation, GDNF treatment prevented mechanical and thermal hyperalgesia as well as reduced spontaneous ectopic discharges in the injured myelinated sensory neurons. Parallel to this, in the damaged DRG GDNF attenuated Na_v1.3 upregulation and partially reversed downregulation of Nav1.8 and Nav1.9 (Boucher et al., 2000).

E. Effect of Inflammatory Mediators and Conditions on Expression and Function of Na⁺ Channels in Primary Sensory Neurons

The first suggestion that TTX-R Na⁺ channels may represent a target for the hyperalgesic action of inflammatory mediators was provided in 1996 when prostaglandin E_2 (PGE₂), adenosine, and serotonin were shown to facilitate the TTX-R current (by enhancing the magnitude of the current, decreasing the activation threshold, and increasing the activation rate), but not the TTX-S one, in DRG neurons (Gold *et al.*, 1996). Activation of protein kinase A increased TTX-R current and induced a leftward shift in its conductance–voltage relationship while protein kinase C activation only enhanced TTX-R current (England *et al.*, 1996; Gold *et al.*, 1998). An involvement of both protein kinase A and protein kinase C activation was revealed in the facilitatory effect of PGE₂ on TTX-R Na⁺ channels by using selective enzyme inhibitors (Gold *et al.*, 1998). A facilitatory action of PGE₂ on TTX-R current was revealed in colonic sensory neurons (Gold *et al.*, 2002). The sensitizing effect of serotonin on TTX-R channels of DRG neurons also involves the adenylyl cyclase–cAMP pathway (Cardenas *et al.*, 2001). Epinephrine (adrenaline) was also demonstrated to potentiate the TTX-R current in sensory neurons, and its effect involved activation of both protein kinase A and C (Khasar *et al.*, 1999). Furthermore, a cAMP-dependent phosphorylation of Na_v1.8 has been shown and the site of phosphorylation has been identified as well (Fitzgerald *et al.*, 1999). In a behavioral study, intrathecal administration of an antisense oligodeoxynucleotide against Na_v1.8 increased the baseline mechanonociceptive threshold and reduced the mechanical hyperalgesia evoked by PGE₂ (Khasar *et al.*, 1998). These data indicate that modulation of TTX-R Na⁺ channels may be involved in the sensitizing and hyperalgesic effects of PGE₂.

In Na_v1.8 knockout mice behavioral evidence was provided for a pronounced deficit in mechanonociception, slight reduction in thermonociception, and delayed development of inflammatory hyperalgesia, indicating an involvement of Na_v1.8 in nociception (Akopian *et al.*, 1999). Furthermore, in these animals NGF-induced thermal hyperalgesia, but not PGE₂-evoked heat hypersensitivity, was reduced (Kerr *et al.*, 2001). In another study, Na_v1.8 null mutant mice showed normal responses to acute noxious stimulation of abdominal viscera but displayed significantly less nociceptive behavior in response to intracolonic capsaicin or mustard oil, which are known to sensitize nociceptors (Laird *et al.*, 2002).

Experimental gastric ulcer was shown to exert a facilitatory effect on TTX-R currents recorded from somata of gastric sensory neurons (Bielefeldt et al., 2002a). Mild gastritis was also found to increase the TTX-R current in gastric spinal sensory neurons (Bielefeldt et al., 2002b). In the carrageenan inflammatory pain model, Nav1.8 expression was upregulated and TTX-R currents in the neurons of the affected DRG were increased (Tanaka et al., 1998). An antisense oligodeoxynucleotide against Na_v1.8 prevented tactile allodynia and thermal hyperalgesia in rats pretreated with complete Freund's adjuvant but failed to alter hyperalgesia evoked by carrageenan (Porreca et al., 1999). Chemical irritation induces bladder hyperactivity that was attenuated following Nav1.8 antisense treatment of rats indicating the involvement of this Na⁺ channel isoform in this model of visceral pain (Yoshimura et al., 2001). TTX-R currents recorded in small afferent neurons of the bladder were also reduced by the treatment showing the role of Na_v1.8 in TTX-R currents. In a study, the expression of all known types of Na⁺ channels was investigated in the carrageenan model (Black et al., 2004). There was an increased expression of Na_v1.3, and Na_v1.7 with a parallel enhancement of TTX-S currents. Nav1.8, but not Nav1.9, was also upregulated and the slowly inactivating TTX-R current was also increased. These data show that TTX-S Na⁺ channels are also upregulated under inflammatory conditions and suggest that they might contribute to inflammatory pain. In another model of inflammation induced by complete Freund's adjuvant, expression of $Na_v 1.8$ was increased in both myelinated and unmyelinated axons while that of $Na_v 1.9$ was decreased in unmyelinated axons (Coggeshall *et al.*, 2004). Both carrageenan and complete Freund's adjuvant can stimulate the production of NGF providing a possible mechanism for $Na_v 1.8$ upregulation in these inflammatory models.

The persistent TTX-R current in small-diameter sensory neurons, attributed to Na_v1.9, was upregulated by guanosine 5'-triphosphate (GTP), which manifested itself as membrane depolarization and spontaneous activity in initially silent neurons (Baker *et al.*, 2003). As several inflammatory mediators act through GTP-binding G-protein–coupled receptors, this mechanism is likely to have relevance for nociceptor hyperexcitability under inflammatory conditions. Nociceptor-specific deletion of the gene for the TTX-S Na_v1.7 channel in mice resulted in increased mechanical and thermal pain thresholds as well as reduced or abolished inflammatory pain responses to formalin, carrageenan, complete Freund's adjuvant, and NGF (Nassar *et al.*, 2004). It is also worth mentioning that primary erythermalgia, a congenital pain syndrome in humans, has been mapped to the Na_v1.7 gene. These data clearly show that both TTX-S and TTX-R Na⁺ channels can be involved in the pathogenesis of inflammatory pain (Lai *et al.*, 2004).

IV. NICOTINIC ACETYLCHOLINE RECEPTORS

As far as we know, the first evidence for a selective excitation of some groups of cutaneous or mesenteric afferents by acetylcholine or nicotine was described by Brown and Gray (1948), who stated: "The impulses probably arise through the direct chemical stimulation of some part of the terminations of the sensory nerves, although we have failed to obtain them from single Pacinian corpuscles." Douglas and Ritchie (1960) showed by using the collision technique that acetylcholine excites cutaneous C-fibers and collisions occur in both the fastest C1 and the slowest C2 elevations of the compound action potential in the cat's saphenous nerve. Chemoreflexes evoked by nicotine were also a kind of indication for a more or less selective action on arterial chemoreceptors and pulmonary receptors that evoke the pulmonary chemoreflex also known as the Bezold-Jarisch reflex (Ginzel, 1975). Acetylcholine evoked pain when it was applied to the exposed blister base (Keele and Armstrong, 1964) and several nicotinic receptor agonists evoked protective reflexes when instilled into the eye of guinea pigs and rats (Jancsó et al., 1961). Nicotinic receptor antagonists blocked these sensory effects, and in the latter study the blocking effect of capsaicin pretreatment was also shown. Close-arterial injection of acetylcholine into the rabbit ear evoked nocifensive reflexes, which were inhibited by nicotinic receptor antagonists but not by

atropine (Juan, 1982). Activation of slowly adapting mechanoreceptors but not other types of low-threshold and high-threshold cutaneous mechanoreceptors was observed in response to close-arterial injection of acetylcholine in the cat (Fjällbrant and Iggo, 1961). In a study on the in vitro rat skinsaphenous nerve preparation, C-mechanoheat-sensitive (C-MH) or C-polymodal nociceptors, C-mechanocold receptors were excited by nicotine in a dose-dependent manner, while muscarine enhanced the noxious heat and mechanical thresholds of the C-polymodal nociceptors. None of the slowly or rapidly adapting A β or A δ afferents or high-threshold mechanoreceptors (A δ -LTM, A δ -HTM) responded to carbachol (Steen and Reeh, 1993; Bernardini et al., 2001). Nicotinic acetylcholine receptors (AChN) have been identified in the rat trigeminal (Liu et al., 1997) or DRG neurons (Boyd et al., 1991). Neonatal capsaicin pretreatment decreased the H³-nicotine binding sites in the central terminals of primary afferent neurons (Roberts et al., 1995). Dorsal root ganglion neurons express multiple subtypes of AChNs. Although the presence of nearly every known AChN subunit was revealed, the largest portion of neurons (77% of large neurons, 32% of small neurons) express the category I-type (α 7-like) subtype of AChN (Genzen *et al.*, 2001).

Interest in cholinergic treatment of painful conditions is related to the discovery of the highly potent poison from the Ecuadorian frog *Epipedobates tricolor* called epibatidine (Spande *et al.*, 1992). This AChN agonist evoked nociceptive reflexes in the rabbit ear preparation, but its short stimulatory effect was followed by long-lasting desensitization (Lembeck, 1999). The pitfalls of this nicotinic cholinergic approach to pain management are related to the wide distribution of nicotinic receptors, which results in a very narrow therapeutic window (Lembeck, 1999; Flores, 2000).

V. SEROTONIN IONOTROPIC RECEPTOR

Serotonin (5-hydroxy-tryptamine, 5-HT) has several G-protein–coupled metabotropic receptor subtypes (5-HT_{1A-F}, 5-HT_{2A-C}, 5-HT₄, 5-HT_{5A,B}, 5-HT₆, 5-HT₇) and one ionotropic receptor (5-HT₃) (Alexander *et al.*, 2006). On the nociceptive and interoceptive nerve endings, activation of the 5-HT₃ receptors elicits action potentials while the metabotropic receptors have in most cases inhibitory effects. 5-HT₇ receptors might be involved in mediation of the serotonin-induced nociception (Meuser *et al.*, 2002). The special role of 5-HT receptors in mediation and prevention of migraine is not discussed here.

The relevance of 5-HT as an endogenous pain-producing substance, which is released from aggregating and activated platelets and from the enterochromaffin cells in the intestines, was known for a long time. When applied to the
exposed blister base it evoked pain followed by tachyphylaxis (Keele and Armstrong, 1964). Serotonin activated C-fibers (Fjällbrant and Iggo, 1961; Beck and Handwerker, 1974; Fock and Mense, 1976). In the isolated rat skin –saphenous nerve preparation, few nociceptors were activated by serotonin, although its sensitizing action on the effect of bradykinin was pronounced (Lang *et al.*, 1990; for further references see Handwerker *et al.*, 1990).

Serotonin depolarized 70% of the C-type small neurons and 47% of the A-type neurons in the bullfrog DRG (Holz *et al.*, 1985). In the rat, DRG neurons in tissue culture serotonin, the 5-HT₃ receptor agonist 2-methyl-5-HT, and phenylbiguanide evoked depolarization (Robertson and Bevan, 1991). On the basis of whole cell membrane current recordings from capsaicin-sensitive DRG neurons, inflammatory mediators including 5-HT induced an inward current in an acidic (pH 6.1) environment. Since capsazepine (10 μ M) inhibited these responses the potentiation of inflammatory mediators was explained by an action on the capsaicin receptor (Vyklicky *et al.*, 1998).

Activation of C-afferents from the mesenteric artery during ischemia in cats was enhanced by intra-arterial injection of 5-HT and diminished by the 5-HT₃ antagonist tropisetron while agonists at 5-HT₂ and 5-HT₁ metabotropic receptors were ineffective (Fu and Longhurst, 1998). In the rat, single unit mesenteric afferents that were sensitive to intraluminal hydrochloric acid were activated by the 5-HT₃ agonist 2-methyl-5-HT, unlike the mechanosensitive afferents. The responses were abolished by the 5-HT₃ antagonist granisetron (Hillsley and Grundy, 1998). Chemonociceptive receptors are more prone to respond to 5-HT than mechanoreceptors and evidence for action on the same nerve terminals, which respond to protons or capsaicin has been shown. Nevertheless, the rat's pelvic/renal R2 capsaicin-sensitive nociceptors did not respond to 5-HT (Szolcsányi, 1984b) indicating some variability of expression of different ion channels in the plasma membrane of various chemonociceptive nerve endings.

In humans, the selective 5-HT₃-receptor antagonist ondansetron reduced pain scores of neuropathic patients in a placebo-controlled crossover study (McCleane *et al.*, 2003).

VI. GLUTAMATE IONOTROPIC RECEPTORS, PROTON-GATED ION CHANNELS (ASIC2, ASIC3), AND P2X PURINOCEPTORS

A. NMDA, AMPA, and Kainate Glutamate Receptors

The three types of ionotropic glutamate receptors comprise the *N*-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate receptor classes, named originally according to their synthetic agonists (Alexander *et al.*, 2004). The role of these ion channels in transmission of nociceptive or pain signals to neurons of the dorsal horn is at present the primary focus of interest in this context. Data indicate, however, that the nociceptive membrane is also equipped with these ion channels. Therefore, this latter scope of the topic will be discussed here briefly.

Application of kainate to isolated dorsal roots produced dose-dependent depolarization (Agrawal and Evans, 1986), and depolarization of DRG neurons mediated by NMDA receptors was reported (Lovinger and Weigh, 1988). Furthermore, expression of AMPA, NMDA, and kainate receptors was shown in the rat DRG neurons (Sato *et al.*, 1993), and approximately 20% of unmyelinated sensory fibers in the glabrous skin of the rat's hindpaw showed NMDA, AMPA, and kainate receptor immunoreactivity (Coggeshall and Carlton, 1998).

Intraplantar injection of NMDA, AMPA, or kainate evoked a nocifensive reaction in the rat (Zhou *et al.*, 1996). In the plantar skin–saphenous nerve preparation glutamate (300 μ M) resulted in sensitization of A δ -MH and C-MH fibers to thermal stimuli (Du *et al.*, 2001). A relatively high concentraton of glutamate is required to evoke nociception and action potentials, but glutamate content in arthritic joints is enhanced and nociceptive effects of glutamate are increased during inflammation (Carlton *et al.*, 2003). Thus, although glutamate ionotropic receptors are in the nociceptive membrane and enhance nociceptive activity, their functional significance needs further support.

B. Proton-Gated Ion Channels

Although the TRPV1 ion channel is also gated by protons, a separate group of acid-sensing ion channels (ASICs) as members of the Na⁺ channel superfamily were identified in nociceptive neurons, which are discussed in Chapter 8 of this volume. From a historical point of view, it needs to be mentioned here that in small neurons of the trigeminal ganglia proton-gated currents proposed to be involved in nociception were described already more than 20 years ago (Krishtal and Pidoplichko, 1981), and the first member of ASICs was cloned in 1997 (Waldman *et al.*, 1997).

C. P2X Receptors

Adenosine 5'-triphosphate (ATP) is painful when applied onto the exposed blister base (Keele and Armstrong, 1964) and elicits inward current in

mammalian sensory neurons (Krishtal *et al.*, 1983). ATP activates both the ionotropic P2X purinergic receptors and the metabotropic (G-protein–coupled) P2Y purinoceptors. Their subtypes, molecular structure, and functional role in the nociceptive membrane are discussed in Chapter 9 of this volume.

VII. INITIATION OF IMPULSES AT NOCICEPTORS

Nociceptive terminal axons comprise a chain of beads or varicosites, which contain accumulated mitochondria and vesicles and are partly or completely uncovered by Schwann cells. In the connecting axons these organelles are less common, neurofilaments appear in internal structure and the axon bundles are tightly bound within the Schwann cell envelope (Szolcsányi et al., 1975; Kruger, 1988, 1996; Heppelmann et al., 1990; Szolcsányi, 1991, 1996b; Cauna, 1966). This structural arrangement allows easier access of noxious chemicals to the free regions of the nociceptive nerve terminal than to the axonal regions as indicated by the fact that capsaicininduced mitochondrial swelling was most pronounced in these free parts of the nerve terminals (Szolcsányi et al., 1975). From a functional point of view, two regions, the generator region and the regenerative region were described for initiation of impulses at the vertabrate mechanoreceptors, which had been shown by direct measurements of potentials from the nerve terminal of the Pacinian corpuscles. The generator region is a nonmedullated special terminal region producing graded potentials known as generator or receptor potentials. The regenerative region has been defined as the site where the propagating impulses are initiated (Gray, 1959; Paintal, 1964). Several measurements on Pacinian corpuscles and less direct approaches on other sensors led to the conclusion that the site of action of drugs is at the regenerative region where less diffusion barrier was predicted in the case of mechanoreceptors (Paintal, 1964; Anand and Paintal, 1988).

On the basis of early studies with capsaicin the concept was proposed that in the case of C-polymodal nociceptors the site of action of noxious chemicals like capsaicin, RTX, or mustard oil is at the generator region (Szolcsányi, 1983, 1988, 1996a,b; Szolcsányi *et al.*, 1991, 1994). Sensory neuropeptides (substance P, CGRP and somatostatin) are released from the activated capsaicin-sensitive nerve terminals (Maggi, 1995; Németh *et al.*, 2003; Szolcsányi *et al.*, 2004). Substance P induces neurogenic inflammation (Jancsó *et al.*, 1968), CGRP is a potent vasodilator mediator (Maggi, 1995), and somatostatin has systemic "sensocrine" anti-inflammatory and analgesic effects (Szolcsányi *et al.*, 2004). Neurogenic inflammation (Jancsó *et al.*, 1968) or neurogenic contraction of the tracheal smooth muscle evoked by vanilloids or piperine (Szolcsányi, 1983) was not inhibited by blocking axonal conduction with local anesthetics and/or TTX. These Na⁺ channel blocking agents abolished regenerative propagated action potentials and prevented the release of sensory neuropeptides evoked by electrical nerve stimulation but did not attenate the release process evoked by capsaicin (Németh *et al.*, 2003; Szolcsányi *et al.*, 2004).

Nociceptive terminal axonal varicosities are equipped with several ion channels gated by noxious chemicals or noxious heat suitable for producing generator potentials (Fig. 7). Opening of ligand-gated cation channels indicated in the figure induces influx of Na^+ and Ca^{2+} into terminal axonal beads of the nociceptive neuron in a graded manner depending on their concentration close to the plasma membrane. Na⁺ influx is mainly responsible for depolarization and Ca²⁺ influx is required for the release of sensory neuropeptides from the capsaicin-sensitive nerve endings. Voltage-gated Na⁺ channel blocking agents did not inhibit the release of neuropeptides induced by depolarization due to opening of the TRPV1 ion channel by capsaicin at the nociceptor. Therefore, there is no evidence for the involvement of voltage-gated Na⁺ channels in the effect of capsaicin at the level of the generator region of nociceptors. The same holds true for omega-conotoxin GVIA, which blocks the N-type voltage-gated Ca²⁺ channels (Maggi, 1995). The P/Q Ca^{2+} channel, however, could participate in the release of sensory neuropeptides since omega-agatoxin TK (250 nM) inhibited the effect of capsaicin (Németh et al., 2003). This concentration of omegaagatoxin TK fully blocked the P-type Ca^{2+} current at the somata, but it was only slightly effective at the axon nerve terminal of rat supraoptic neurons (Fischer and Bourque, 1995). Therefore involvement of both P-type and Q-type Ca^{2+} channels in the capsaicin-induced release of mediators should be considered. Actions of substances on ion channels in nerve terminals might be profoundly different from those on cell bodies of DRG neurons or transfected cells since, for example, endoplasmic reticulum or Golgi apparatus which might contain a significant portion of the TRPV1 ion channels (Tóth et al., 2004) are not present in the nerve terminals.

Measurement of membrane currents and intracellular Ca²⁺ concentration in sensory nerve terminals in the cornea of the rat and guinea pig provided the first elegant evidence for the operation of the generator region of capsaicin-sensitive nociceptors (Brock *et al.*, 1998, 2001; Gover *et al.*, 2003). Figure 6A-D shows Ca²⁺ transients evoked by 1 μ M capsaicin in an individual epithelial corneal nerve terminal measured with the fluorescent dye loading technique. Response to capsaicin of a control nerve terminal (A), response to capsaicin in the presence of saxitoxin (B), or in the presence of L-type voltage-gated Ca²⁺ channel blocking agent nifedipine (C) as well as in the presence of the TRPV1 receptor antagonist capsazepine (D) are seen in the



FIGURE 6 Ca²⁺ transients evoked by capsaicin in corneal nerve terminals. (A) Response of an individual nerve terminal to 1 μ M capsaicin. (B) Response of an individual nerve terminal to 1 μ M capsaicin after a 30 min preincubation with 4.1 μ M saxitoxin (STX). (C) Response of an individual nerve terminal to 1 μ M capsaicin after a 20 min preincubation with 20 μ M nifedipine. (D) The response to capsaicin was completely blocked by a 35 min preincubation with 100 μ M capsaicin application. Images were 400 ms exposures acquired at 0.2 Hz (A–C) or 1 Hz (D). (E) Averages of electrically evoked (upper trace) and spontaneously occurring (lower trace) nerve terminal impulses recorded from a corneal mechanonociceptor. A, B, C, and D are reprinted from Gover *et al.* (2003) and E from Brock *et al.* (1998).

figure. The voltage-gated ion channel blocking agents did not attenuate capsaicin-induced Ca²⁺ transients, in fact the Na⁺ channel blocking saxitoxin induced a significant enhancement. Carlos Belmonte's and James Brock's group in Alicante made a series of recordings of membrane potential changes in corneal polymodal receptors, mechanoreceptors and cold receptors. They observed differences among the responses of these receptors.



FIGURE 7 Schematic representation of ion channels located in peripheral terminals of cation nociceptive primary afferent neurons. AchN, nicotinic acetylcholine receptor; ASIC, acid-sensing ion channels; GLU, ionotropic glutamate receptors; 5-HT₃, ionotropic serotonin receptor; KC, K⁺ channels; P2X, ionotropic purinoceptors; TRPV, transient receptor potential vanilloid receptor; TTX-R, tetrodotoxin-resistant voltage-gated Na⁺ channel; TTX-S, tetrodotoxin-sensitive voltage-gated Na⁺ channel; VGCC, voltage-gated Ca²⁺ channel (types N and P/Q). For other abbreviations see text.

Furthermore, they described differences between the shapes of nerve terminal impulses evoked by an antidromic electrical stimulus (Fig. 6E upper record) and that occurred spontaneously on the same nerve terminal (Fig. 6E lower record). One should note the slow rise of spontaneous nerve terminal impulses in the figure, which might be generator potentials followed by the major increase that is due to opening of TTX-R Na⁺ channels (Brock et al., 1998). Nevertheless, owing to technical difficulties, recording generator potential from nociceptor nerve terminals, which cannot be inhibited by Na⁺ channel blocking agents, has not been succeded. It is more likely that similarly as in the axonal initial segment at the somata of neurons (Garrido et al., 2003) there is formation of clusters of Na⁺ channels for initiation of propagated impulses at the axon-varicosity transitions. Figure 7 shows in a schematic way the differences between the variocosity/generator region and axon/regenerative region parts proposed to explain the actions induced by chemonociceptive stimuli, which act by opening different ion channels. A chain of triggering regenerative spikes between generator regions could modify the output signal, which finally is propagated to the central nervous system. This might explain the difference between the thermodependence of the capsaicin effects observed at the level of somata in vitro (Voets et al.,

2004) and nerve terminals *in vivo* (Figs. 1 and 2). Most of the cation channels depicted in the figure are expressed in several types of neurons, and their agonists activate other systems beyond nociceptors. Among sensory afferents the capsaicin-induced activation of single fibers is highly selective and there is no evidence that under physiological conditions TRPV1 is expressed and participates in responses of nonnociceptive sensors except the warmth sensors. Thus TRPV1, TRPV2, and TRPV3 ion channels will be useful markers for the transducer role of ion channels in initiation of impulses at nociceptors and has opened up new perspectives for development of the first analgesic agent, which acts selectively on nociceptors.

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CHAPTER 3

The Nociceptive Membrane: Historical Overview

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I. INTRODUCTION

A. Nociceptors

Nociceptors are primary afferent neurons that signal tissue damage or the threat of tissue damage (Sherrington, 1906; Burgess and Perl, 1973). Nociceptors are found in the skin and subcutaneous tissue, muscle, joints,

and viscera (Schmidt *et al.*, 1987; Belmonte and Cervero, 1996; Kruger, 1996; Kumazawa *et al.*, 1996; Willis and Coggeshall, 2004). They also supply the meninges (Andres *et al.*, 1987), although they do not intrude into central nervous system (CNS). Nociceptors have unencapsulated terminals in the tissue that they innervate (Fig. 1). Generally, the axons of cutaneous nociceptive afferents are either finely myelinated (A δ) or unmyelinated (C) fibers (Erlanger and Gasser, 1937; Willis and Coggeshall, 2004). However, some cutaneous nociceptors are supplied by large myelinated (A β) fibers (Georgopoulos, 1976; Meyer *et al.*, 1991; Treede *et al.*, 1998). Like cutaneous nociceptors, visceral nociceptors are called A δ - and C-fibers (Sengupta *et al.*, 1990). Nociceptors that supply muscle or joints are also finely myelinated and unmyelinated afferents, but a different nomenclature is used; these are referred to as group III and group IV fibers (Lloyd and Chang, 1948; Schaible and Grubb, 1993; Willis and Coggeshall, 2004).



FIGURE 1 Terminals of nociceptors. (A) Several "free endings" of an A δ mechanical nociceptor in the skin. Note the association of Schwann cells with even the tips of the endings. Abbreviations: A, axon; BL, basal lamina; K, keratinocytes; My, myelin sheath; Sw, Schwann cell. Reprinted from Perl (1984). (B) Terminals of a group III and a group IV joint nociceptor. Magnified longitudinal and cross-sectional views of the terminals are shown at the right. Note that in the axonal bead region (lower right panel), a Schwann cell covers much of the surface of the sensory axon, but there is a bare area of axonal membrane that may serve as a receptor site. Reprinted from Schaible and Schmidt (1996).

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B. Morphology of Nociceptors

Although the terminals of nociceptors are often described as "free nerve endings" (Sherrington, 1906), actually much of the terminal membrane of nociceptors is covered by Schwann cells (Fig. 1). However, there are membrane areas that are exposed to the extracellular space (Fig. 1B) (Kruger et al., 1981; Perl, 1984; Heppelmann et al., 1990, 1995). Presumably, these exposed areas of the surface membranes of nociceptors are the sites where the transduction of nociceptive stimuli occurs. For this purpose, these membrane areas would contain special sensory transduction molecules that allow the selective activation of these primary afferents by pain-provoking stimuli (see later). Such an arrangement would be consistent with the statement by Sherrington (1906) that "the absence of any highly evolved specialized end-organ in connexion with (the nociceptive endings) may explain their fairly equal amenability to an unusually wide range of different kinds of stimuli...," "a group of excitants which has in relation to the organism one feature common to all its components, a nocuous character." The ability of an individual nociceptor to respond to different types of noxious stimuli would depend on the presence or absence of particular transduction molecules in its surface membrane.

The cell bodies of primary afferent nociceptive neurons are located in dorsal root and cranial nerve ganglia, such as the trigeminal ganglion, and the axons of these nociceptors convey information about tissue damage to the CNS by way of dorsal roots or cranial nerves (Willis and Coggeshall, 2004). This activity can lead to the sensation of pain, provided that there is sufficient input to produce adequate spatial and temporal summation (Konietzny *et al.*, 1981; Van Hees and Gybels, 1981; Ochoa and Torebjörk, 1989). In addition to pain sensation, nociceptors can also evoke a number of nociceptive reactions, including flexor withdrawal reflexes, endocrine changes, and motivational-affective responses such as arousal and aversive behavior (Sherrington, 1906; Hardy *et al.*, 1952; Melzack and Casey, 1968; Bonica, 1990; Price, 1999; Casey and Bushnell, 2000).

The dorsal root ganglion (DRG) cells that give rise to C-fibers are smaller in size than those that give rise to large-myelinated axons (Lawson *et al.*, 1984; Lawson and Waddell, 1991). However, DRG cells with A δ fibers are intermediate in diameter and overlap the size ranges of A β - and C-fibers (Lawson and Waddell, 1991). Although small DRG cells are often associated with C or A δ fibers, this does not mean that a given small DRG cell is necessarily a nociceptor (Willis and Coggeshall, 2004). Some C-fibers are sensitive mechanoreceptors (Iggo, 1960; Bessou *et al.*, 1971) and others are warm thermoreceptors (Hensel *et al.*, 1960; Iriuchijima and Zotterman, 1960). Similarly, some A δ fibers are sensitive mechanoreceptors (Burgess and Perl, 1967) and some are cold thermoreceptors (Willis and Coggeshall, 2004). The identification of putative nociceptors requires the demonstration of nociceptive responses or the presence of one or more nociceptive markers such as substance P (SP) content or membrane receptors that are restricted to nociceptors such as capsaicin (TRPV1) receptors.

C. Peripheral Sensitization of Nociceptors

Nociceptors are not only excited by intense stimuli, but they can also be sensitized during the process of inflammation by the actions of a variety of inflammatory mediators on receptors contained in the surface membranes of the nociceptors (Walker et al., 1995; Coggeshall and Carlton, 1997; Burnstock, 2000; Willis and Coggeshall, 2004). These receptors are coupled to intracellular signal transduction cascades (Guenther et al., 1999; Kress and Guenther, 1999), and protein kinases in these cascades can phosphorylate the transduction molecules or ion channels in the membranes of nociceptors. Phosphorylation of these membrane proteins may result in a lowered threshold to stimuli, a process called "peripheral sensitization" (Bessou and Perl, 1969; Lynn and Carpenter, 1982; Häbler et al., 1990; England, et al., 1996; Gold et al., 1996b, 1998; Nicol et al, 1997b; Khasar et al., 1998; Lopshire and Nicol, 1998; Djouhri and Lawson, 1999; Fitzgerald, et al., 1999). For example, some nociceptors called "silent nociceptors" or "mechanically insensitive afferents" are unresponsive to mechanical stimuli unless they are first sensitized, but when sensitized they respond even to very weak mechanical stimuli (Fig. 2) (Schaible and Schmidt, 1983a,b, 1985; Handwerker et al., 1991; Meyer et al., 1991; Kress et al., 1992; Davis, et al., 1993; Schmidt et al., 1995, 2000; Treede et al., 1998).

Sensitized nociceptors are thought to be responsible for the primary mechanical allodynia (pain caused by normally innocuous stimuli) and hyperalgesia (enhanced pain in response to a painful stimulus) that may occur following injury (Meyer and Campbell, 1981; LaMotte *et al.*, 1982, 1983; Merskey and Bogduk, 1994).

D. Transduction Molecules and Ion Channels in Nociceptors

The molecules in the surface membranes of nociceptors that are responsible for the transduction of nociceptive stimuli are present not only on the peripheral terminals, but they may also be found along the length of the axons and in the cell bodies of the nociceptors in sensory ganglia (Coggeshall



FIGURE 2 Sensitization of a group III nociceptor that supplied the knee joint in a cat. Initially, the nociceptor failed to respond to flexion of the knee. However, by 87 min after injection of the joint capsule with kaolin, the afferent began to respond to knee flexion, and the response increased progressively over the next hour and a half. The sensitized response was then maintained for a long period of time. Reprinted from Schmidt (1996).

and Carlton, 1997; Szallasi and Blumberg, 1999; Karai *et al.*, 2004b; Willis and Coggeshall, 2004). This allows the study of the transduction mechanisms responsible for nociceptive responses in cultures of isolated putative nociceptive DRG cells (Baccaglini and Hogan, 1983) or in other *in vitro* preparations such as the isolated skin–nerve preparation (Reeh, 1986). Another important aspect of the membrane properties of nociceptors is the ion channels that are responsible for the conduction of nerve impulse activity in the axons of these nerve fibers. Some of these ion channels are found specifically in nociceptors and not in other types of primary afferent fibers, and they may be up- or downregulated following injury.

II. NOCICEPTIVE TRANSDUCTION

Pain can be evoked by any of several kinds of stimuli, including intense mechanical, thermal, or chemical stimuli (Willis and Coggeshall, 2004). Noxious mechanical stimuli include mechanical compression of tissue and application of a pointed object such as a pin. Noxious thermal stimuli include both noxious heat and noxious cold. Examples of noxious chemical stimuli are intradermal injection of capsaicin or formalin and topical application of mustard oil to the skin. A given type of nociceptor may respond preferentially to one or more modalities of noxious stimuli (Davis *et al.*, 1993; Schmidt *et al.*, 1995). For example, $A\delta$ mechanical nociceptors respond to noxious intensities of mechanical stimulation but not to noxious thermal or chemical stimuli (Burgess and Perl, 1967), whereas C polymodal nociceptors can be activated by noxious mechanical, thermal, or chemical



FIGURE 3 Ways in which nociceptors can be activated, sensitized, or modified. At the top, external stimuli, such as heat, mechanical, or chemical stimuli, impinge on transducers or receptors in the nociceptor's surface membrane, such as VR1, mDEG, or P2X3, causing a receptor potential, activation of voltage-gated sodium channels, and nerve impulse conduction. Input to the CNS leads to pain sensation. The activity in the nociceptors may produce "autosensitization" through signal transduction mechanisms. In the middle, the nociceptor terminals are modulated by the action of agonists (e.g., PGE_2 or bradykinin) that can produce peripheral sensitization (heterosensitization) by interactions with surface membrane receptors [e.g., prostaglandin (EP) or bradykinin (BK) receptors], leading to activation of signaling pathways (e.g., PKA or PKC). Sodium channels (SNS/PN3) may be upregulated. In the lower drawing, the nociceptor may be modified by a change in gene expression. For example, an increase in gene expression may result in more VR1 or SNS/PN3 channels, resulting in abnormal sensitivity of the afferent. Or there may be a phenotypic switch, such as the expression of SP or BDNF in A fibers. Release of these substances as neurotransmitters may mimic the action of C-fibers. Finally, loss of C-fibers by denervation may provoke neuropathic changes. Reprinted from Woolf and Salter (2000).

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stimuli (Bessou and Perl, 1969). The capacity of nociceptors to respond selectively to different modalities of noxious stimuli depends on the transduction molecules that are present in the surface membranes of these neurons (Davis *et al.*, 1993; cf., Iriuchijima and Zotterman, 1960). Selectivity also depends on whether or not the nociceptor is sensitized or is changed because of an alteration in gene expression (Fig. 3) (Woolf and Salter, 2000). Our knowledge about nociceptive transduction molecules is still fragmentary but is increasing.

A. Transduction of Noxious Mechanical Stimuli

The membrane proteins that are responsible for the transduction of noxious mechanical stimuli appear to belong to the amiloride-sensitive degenerin/epithelial sodium channel (DEG/ENaC) family of proteins that was first described in Caenorhabditis elegans (Fig. 4A and B) (Driscoll and Chalfie, 1991; Huang and Chalfie, 1994; Waldmann and Lazdunski, 1998). This family of channels shares the following properties: they have Na^+ permeability; they are inhibited by the diuretic amiloride; and they are not voltage-gated (Waldmann and Lazdunski, 1998). Proteins in this family form cation channels not only in mechanoreceptors of C. elegans and other invertebrates but also in mechanoreceptors of mammals (García-Añoveros et al., 1997, 2001; Drummond et al., 2000; Price et al., 2000; Kellenberger and Schild, 2002; Welsh et al., 2002). Several theories concerning how these channels are gated have been proposed. One idea is that an increased tension within the lipid bilayer of the surface membrane of mechanoreceptors causes the channel to open (Fig. 4C). Another possibility is the release of a ligand, such as ATP, which then excites the afferent (Fig. 4D) (Hamill and Martinac, 2001; Welsh et al., 2002). A third proposal is that these mechanically sensitive channels are fastened to the extracellular matrix and cytoskeleton in such a way that mechanical tension across the surface membrane gates the channels (Fig. 4E) (García-Añoveros and Corey, 1997; Welsh et al., 2002). Other channels in this protein family are ligand-gated (by protons or by a peptide).

A mechanically gated whole-cell current has been recorded in patch clamp experiments on rat DRG cells (Fig. 5). This current could be blocked by gadolinium or by benzamil (an analog of amiloride) and was suggested to be mediated by a member of the DEG/ENaC family (McCarter *et al.*, 1999). The current was observed in DRG neurons of all sizes, including neurons that could be sensitized by prostaglandin E_2 and that therefore were putative nociceptors. No such current was seen in sympathetic ganglion cells treated in the same way. It has been proposed that BNaCl α might be the mechanical



FIGURE 4 (A) Model showing the tetrameric structure of an ENaC channel. (B) Model of the mechanoreceptor channel in *C. elegans*. Note the large extracellular domain and the linkages to the extracellar matrix (including MEC-5 collagen) and cytoskeletal elements, MEC-12 and MEC-7 microtubules. Reprinted from Kellenberger and Schild (2002). (C–E) Possible mechanisms by which mechanosensitive channels might respond to a mechanical stimulus, including tension developed in the surface membrane lipid bilayer, release of a ligand that binds to receptors on the adjacent membrane, and tethering of the channel to the extracellular matrix and the cytoskeleton. Reprinted from Welsh *et al.* (2002).

transducer both in sensitive mechanoreceptors and in A δ mechanical nociceptors (García-Añoveros *et al.*, 2001). However, although deletion of the gene for BNaC1 reduces the responsiveness of many sensitive mechanoreceptors, such a knockout does not affect the responses of other sensitive



FIGURE 5 Responses of a sensory neuron to mechanical stimuli. (A) The top trace is a monitor of the position of a piezoelectric device that applied a mechanical stimulus to a DRG cell during a whole cell patch clamp experiment. The probe moved about 50 μ m at 300 μ m/s. The middle trace shows action potentials evoked in the neuron by the mechanical stimulus while recording in the current clamp mode. The resting potential was -59 mV. The bottom trace shows the transient and sustained inward currents produced by the same stimulus while the neuron was voltage clamped at -60 mV. (B) Inward currents produced by a hydraulic pressure stimulus produced by puffing 3-s jets of bath solution using pressures of 2.5, 5, 15, and 45 psi. The graph shows the relationship between peak current amplitude and pressure. Reprinted from McCarter *et al.* (1999).

mechanoreceptors or of A δ and C mechanical nociceptors, suggesting that other proteins are also involved in the formation of mechanically sensitive channels in sensory neurons (Price *et al.*, 2000).

B. Transduction of Acidic Stimuli

Acid-sensitive ion channels (ASICs) also belong to the DEG/ENaC family of proteins. As already mentioned, ASICs are cation channels that respond to protons. The ASICs include the DRASIC (or ASIC3) channel (Waldmann and Lazdunski, 1998). DRASIC is found in both large and small DRG neurons, including small sensory neurons that express SP, as well as in the terminals of sensitive mechanoreceptors and in some free nerve endings in the skin (Price *et al.*, 2001). The sensory neurons that contain SP and many that give rise to axons with free endings are likely to be nociceptors (Lawson *et al.*, 1997). Curiously, when this channel is knocked out in mice, rapidly adapting but not slowly adapting, sensitive mechanoreceptors develop enhanced responses. By contrast, nociceptors show reduced responses to noxious mechanical, heat, and acid stimuli (Price *et al.*, 2001). On the other hand, knockout of the ASIC2 and ASIC3 channels does not result in the loss of mechanically activated currents in rat DRG cells (Drew *et al.*, 2004).

Cold temperatures increase the current produced by activation of ENaC (but not BNC1, ASIC, or DRASIC) and enhance the currents produced by acid stimulation in all of these channels, perhaps explaining the effects of temperature on the responses of tactile Merkel cell (SA I) endings (Askwith *et al.*, 2001; Willis and Coggeshall, 2004).

Lowered pH is also detected by TRPV1 receptors, as discussed in the next section.

C. Transduction of Noxious Thermal and Chemical Stimuli

The responses of nociceptors to noxious thermal and chemical stimuli depend in part on a family of proteins called transient receptor potential (TRP) receptors found originally in *Drosophila* photoreceptors (Montell *et al.*, 1985). There are at least six members of the TRP family that are sensitive to temperature (thermoTRPs, Fig. 6) (Patapoutian *et al.*, 2003; Cortright and Szallasi, 2004; Voets *et al.*, 2004). Four TRP channels that belong to the TRPV subfamily respond to heat. TRP receptors can also respond to a variety of chemical stimuli.

1. Vanilloid (or TRPV) Receptors

Capsaicin (8-methyl-N-vanillyl-6-nonenamide), the active ingredient in chili peppers (Fig. 7A, left), produces burning pain or itch in human subjects (Szolcsányi, 1993; Szallasi and Blumberg, 1999) by activating polymodal C and mechano-heat A δ cutaneous nociceptors (Szolcsányi et al., 1988). Capsaicin excites these sensory neurons by opening nonselective cation channels that allow influx of Na⁺ and Ca²⁺ ions into these sensory neurons, resulting in their excitation (Marsh et al., 1987; Wood et al., 1988). Ca²⁺ entry is not primarily through voltage-gated Ca^{2+} channels, since blockers of these channels do not impair the influx of Ca²⁺ in response to capsaicin (Wood et al., 1988). Resiniferatoxin is a potent agonist of capsaicin receptors (Fig. 7A, right). Capsaicin does not activate A mechanoreceptors or C mechanoreceptors, $A\delta$ mechanical nociceptors or cold receptors (Fitzgerald, 1983; Szolcsányi et al., 1988). The activity of warm receptors increases and that of cold receptors decreases following capsaicin administration, but these effects are apparently secondary to the increased temperature that results from vasodilation in the skin (see later), rather than due to a direct action on thermoreceptors.

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FIGURE 6 Roles of thermo TRP receptors. A series of DRG cells innervating the skin is shown diagrammatically. Each contains a particular TRPV receptor or a combination of two TRPV receptors, as indicated. Various thermal stimuli that would activate the DRG cells are shown at the left. These include capsaicin (shown by drawing of a chili pepper), heat, menthol (shown by mint leaves), cool and cold. Reprinted from Patapoutian *et al.* (2003).

Capsaicin plays a sensory role by activating nociceptors, but it also has an efferent function by initiating neurogenic inflammation. Activation of vanilloid receptors causes the local release of tachykinins, including SP, and other peptides, such as calcitonin gene-related peptide (CGRP), from the terminals of peptidergic nociceptors (Fig. 8) (Holzer, 1988; Szolcsányi, 1988; Geppetti and Holzer, 1996). SP produces plasma extravasation and neurgenic edema by an action on postcapillary venules, and CGRP produces vasodilation (flare) by relaxing arterioles (Holzer, 1988; Geppetti and Holzer, 1996; Willis and Coggeshall, 2004).

Flare and neurogenic edema spread a considerable distance from a site of injury. For example, intradermal injection of $100 \ \mu g$ of capsaicin into the skin of a human volunteer results in a flare with an average area of $31 \ \text{cm}^2$ (Simone



FIGURE 7 (A) Structures of two exogenous vanilloid compounds that activate TRPV1: capsaicin and resiniferatoxin. Reprinted from Szallasi and Blumberg (1999) with permission. (B) Structures of several proposed endogenous vanilloid-like compounds (endovanilloids) that may serve as natural ligands for TRPV1. These include 15-HPETE, 12-HPETE, anandamide, NADA and *N*-oleoyldopamine. Reprinted from Van der Stelt and Marzo (2004).

et al., 1989). The axon reflex is a mechanism that has been proposed to explain this spread of neurogenic inflammation (Fig. 8) (Lewis, 1927; cf., Lynn, 1988; Schmelz *et al.*, 1997; Schmidt *et al.*, 1997). This is a plausible mechanism in humans if the flare results from the activation of the "itch-receptors" described by Schmelz *et al.* (1997), since the terminal arborizations of these receptors are very large. However, in laboratory animals, flare often spreads well beyond the terminal axonal arbor of the nociceptors that release CGRP. There is now strong evidence that neurogenic edema and flare spread by means of dorsal root reflexes (DRRs), at least in rats (Fig. 9) (Sluka and Westlund, 1993; Sluka *et al.*, 1993; Rees *et al.*, 1994, 1995, 1996; Lin *et al.*, 1999, 2000).



FIGURE 8 Spread of flare by means of axon reflex. In (A), a noxious stimulus, such an intradermal injection of capsaicin, is applied at the site on the skin indicated by the arrow (bottom left). Peptides released from the nearby terminal of a nociceptor are indicated by filled circles. The release of peptides occurs because of direct activation of capsaicin-sensitive receptors on the terminal. The axon may also be stimulated enough to exceed threshold for nerve impulse conduction, leading to propagation of action potentials into other branches of the nociceptive terminal, as indicated by the bent arrow. Invasion of these terminals results in release of peptides at some distance from the original stimulus. This distance will depend on the structure of the terminal arborization, which varies across species. The inset shows a central dark region that represents the flare response. In (B), a local anesthetic was applied to the skin; this blocked the propagation of the axon reflex and prevented the spread of the flare. The inset shows only the local enhancement of blood flow. In (C), capsaicin receptors were desensitized by prior application of capsaicin, and so noxious stimulation is ineffective in releasing peptides and no reddening of the skin is seen. Reprinted from Jancsó *et al.* (1967).



FIGURE 9 Spread of flare by axon reflex versus dorsal root reflex. (A) shows how flare might be spread by an axon reflex (see also Fig. 8). In this case, capsaicin is injected into the skin, and after propagation of nerve impulse activity into collaterals of the activated nociceptor terminals, peptides, such as CGRP, are released, causing vasodilation that can be measured by a laser Doppler blood flow probe. Afferent activity also enters the spinal cord over dorsal roots and activates ascending nociceptive pathways, causing pain. In (B), the capsaicin injection activates nociceptive afferents, and afferent activity reaches the spinal cord by way of dorsal roots. In addition to triggering ascending signals that result in pain, the afferent input also activates GABAergic inhibitory interneurons, which cause primary afferent fibers, including C-fibers, and these release peptides in the periphery, resulting in neurogenic inflammation. Evidence that this happens in rats is that flare is blocked when the peripheral nerve is interrupted at a proximal site or following dorsal rhizotomy, and further, that administration of antagonists of glutamate or GABA_A receptors in the spinal cord will also block the flare. None of these procedures would be expected to affect axon reflexes. Reprinted from Willis (1999).

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a. VR1 or TRPV1. The first TRPV receptor to be investigated responds to capsaicin and other vanilloid chemicals such as the ultrapotent agonist of capsaicin, resiniferatoxin (Fig. 7A), and so it was initially called vanilloid receptor 1 (VR1) (Buck and Burks, 1986; Szallasi and Blumberg, 1999; Caterina and Julius, 2001; Cortright and Szallasi, 2004). VR1, now known as TRPV1, is found in more than 50% of DRG cells, mostly in small- to medium-sized ones (Guo *et al.*, 1999). These give rise to many capsaicinsensitive C-fiber nociceptors and also some capsaicin-sensitive A δ nociceptors (Nagy *et al.*, 1983; Baumann *et al.*, 1991; LaMotte *et al.*, 1992). A subpopulation of DRG cells, which contains TRPV1, colocalizes peptides such as SP and CGRP. However, a larger subpopulation of sensory neurons shows colocalization of TRPV1 with the lectin, IB4, and P2X₃ receptors (Guo *et al.*, 1999).

TRPV1 can be activated by several types of noxious stimuli, including capsaicin and related chemical substances, noxious heat and protons but not by noxious mechanical stimuli or cold (Fig. 10) (Caterina *et al.*, 1997, 2000; Tominaga *et al.*, 1998; Vyklický *et al.*, 1998; Davis, *et al.*, 2000; Julius and



FIGURE 10 Diagram of the sites on the TRPV1 molecule that are activated by vanilloids, such as capsaicin, noxious heat, reduced pH, and eicosanoids such as anandamide. The ability of agents that sensitize nociceptors, such as bradykinin and prostaglandins, is mediated through activation of protein kinases, which in turn phosphorylate TRPV1. Reprinted from Cortright and Szallasi (2004).

Basbaum, 2001). Furthermore, a lowered pH greatly enhances the membrane currents produced in sensory neurons by capsaicin application (Petersen and LaMotte, 1993). One view is that heat is the stimulus required to open TRPV1 channels; capsaicin and low pH may reduce the heat threshold sufficiently for normal room temperatures to open the channels (Tominaga *et al.*, 1998; Szallasi and Blumberg, 1999). Mice lacking TRPV1 respond normally to noxious mechanical stimuli, but the mice have difficulty in detecting noxious heat stimuli, and they develop little thermal hypersensitivity during inflammation (Caterina *et al.*, 2000; Davis *et al.*, 2000). Inconsistent with the terminology, TRPV receptors other than TRPV1 do not respond to capsaicin.

TRPV1 gating is inhibited by an interaction with intracellular phosphatidylinositol-4,5 bisphosphate [PtdIns(4,5)P₂]. Bradykinin and nerve growth factor (NGF) enhance TRPV1 activity by removing this inhibition by PtdIns (4,5)P₂ (Chuang *et al.*, 2001). NGF also stimulates an increase in TRPV1 expression in DRG neurons (Winston *et al.*, 2001). This results from activation of the p38 MAPK pathway (Ji *et al.*, 2002).

There has been considerable interest in the identification of endogenous ligands for TRPV1 ("endovanilloid" compounds) (Di Marzo et al., 2002; Van der Stelt and Di Marzo, 2004). These include several metabolic products of arachidonic acid (Fig. 7B), including the endocannabinoid, anandamide (Zygmunt et al., 1999; Smart et al., 2000), N-arachidonyldopamine (NADA) (Huang et al., 2002), N-oleoyldopamine (Chu et al., 2003), and lipoxygenase products of arachidonic acid such as 12S- and 15S-HPETE (Hwang et al., 2000; Shin et al., 2002). The observation that acid activates nociceptors and produces burning pain in humans (Steen et al., 1992, 1995a) and that protons result in membrane currents in DRG cells similar to the currents evoked by capsaicin, led to the idea that protons might activate capsaicin receptors (Bevan and Yeats, 1991; Bevan and Geppetti, 1994; McLatchie and Bevan, 2001). Bradykinin, acting at B2 receptors, has been found to stimulate the production of 12-HPETE by activation of the 12-lipoxygenase pathway. The 12-HPETE in turn acts as an agonist of TRPV1, as shown by the ability of capsazepine or of an inhibitor of the 12-lipoxygenase pathway to prevent the action of bradykinin (Shin et al., 2002).

b. VRL1 or TRPV2. A molecule related to TRPV1, known as vanilloid receptor-like 1 (VRL1) or TRPV2 (Fig. 6), responds to higher intensities of noxious heat (threshold of about 52°C) than does TRPV1 (threshold of 43°C) (Caterina *et al.*, 1999; Patapoutian *et al.*, 2003). Although VRL1 is called a vanilloid receptor because of structural homology to TRPV1, in actuality TRPV2 neither responds to capsaicin nor to acid or moderate
intensities of heat (Caterina *et al.*, 1999). Like TRPV1, TRPV2 receptors are found in sensory neurons with axons of either A δ - or C-fiber caliber (Ma, 2001, 2002). Unlike in rats, DRG cells in mice that bind isolectin B4 (IB4) are nociceptors that are responsive to heat, and yet these nociceptors do not express TRPV1 and most do not express TRPV2 either (Woodbury *et al.*, 2004). Furthermore, heat-responsive nociceptors can be found in TRPV1null mice, and these neurons have normal heat thresholds, suggesting that other heat-sensitive molecules also help mediate responses to heat.

c. TRPV3 and TRPV4. Two other TRPV receptors, TRPV3 and TRPV4, respond to warm temperatures [\sim 34°C to 38°C and \sim 27°C to 34°C, respectively (Fig. 6)] (Patapoutian *et al.*, 2003). However, TRPV3 responses continue to increase as the temperature is increased into the noxious range. Furthermore, TRPV3 responses are sensitized by repeated heat stimuli, suggesting that TRPV3 might contribute to nociception (Patapoutian *et al.*, 2003). These TRP receptors are found in sensory neurons and also in cutaneous keratinocytes (Chung *et al.*, 2003, 2004), suggesting that keratinocytes may serve as thermosensory cells. They could activate sensory nerve terminals by releasing a soluble messenger such as ATP (Chung *et al.*, 2003). However, such an action has not been established (Patapoutian *et al.*, 2003).

d. TRPM8 and ANKTM1. Transduction in cold receptors and cold nociceptors involves other members of the TRP family (Voets et al., 2004). TRPM8 responds to cooling ($<28^{\circ}$ C) and to menthol (Fig. 6) (McKemy et al., 2002; Peier et al., 2002; Nealen et al., 2003), which is well known to cause a cool sensation (Patapoutian et al., 2003) and to activate cold receptors (Hensel and Zotterman, 1951). TRPA1, also called ANKTM1, for "ankyrin repeat/transmembrane-containing ion channel" (Fig. 6), is insensitive to menthol but is activated at noxious cold temperatures ($<18^{\circ}$ C), according to Story et al. (2003). This thermal sensitivity has been disputed by Jordt et al. (2004), who found that primary sensory neurons with ANKTM1 receptors respond to isothiocyanate compounds, such as those found in mustard oil and other pungent plant derivatives (wasabi, horseradish), as well as to tetrahydrocannabinol, but not to cold. However, Bandell et al. (2004) observed that ANKTM1 receptors do respond to noxious cold, although they also confirmed that these receptors are activated by pungent substances, such as those present in mustard oil, cinnamon oil, wintergreen oil, ginger, and clove. The most specific activator of ANKTM1 was found to be cinnamaldehyde. Bradykinin produces a similar activation. This is explained by the fact that most ANKTM1-positive DRG cells also express

bradykinin receptors. Phospholipase C signaling is important for the activation of ANKTM1 receptors.

e. Other Temperature-Sensitive Molecules in Sensory Neurons. Other molecules may prove to contribute to thermal sensitivity. For example, a heat-responsive K^+ channel called TREK-1 is highly expressed in DRG cells, as well as in the hypothalamus (Maingret *et al.*, 2000). Warm temperatures open these channels, which would lead to hyperpolarization, and cooling would result in depolarization and presumably trigger action potentials.

The activity of some members of the DEG/ENaC family of sodium channels is potentiated by cold (Askwith *et al.*, 2001).

In knockout mice lacking the ATP-gated purinergic channel, $P2X_3$, spinal interneurons respond normally to noxious heat but show a deficit in their responses to warm temperatures. In addition, there are deficits in both phases of the formalin test. Curiously, the knockout results in an enhancement of thermal hyperalgesia during chronic inflammation (Souslova *et al.*, 2000).

2. Regulation of TRPV1

a. Voltage Dependence. Temperature sensing by TRP family cation channels is voltage dependent (Voets *et al.*, 2004). The activation curve of TRPM8 channels is shifted dramatically leftward by lowered temperatures or menthol. Similarly, the activation curve of TRPV1 is shifted leftward by increased temperature and by capsaicin. In both cases, the thermal activation curves are shifted toward membrane potentials that are seen under physiological conditions.

b. Modulation by Inflammatory Mediators. The activity of TRPV1 can be modulated by neurotransmitters or inflammatory substances that activate receptors on the surface membranes of TRPV1-containing nociceptors. For example, activation of NK₂ tachykinin receptors by SP can secondarily affect TRP channel receptors expressed in HEK293 cells through activation of the phospholipase C signaling pathway (Oh *et al.*, 2003). ATP acting on P2Y₂ receptors also modulates TRPV1 receptor activity through a PKC pathway (Moriyama *et al.*, 2003), and ATP-induced thermal hyperalgesia is abolished in TRPV1-null mice but not in P2Y₁ knockout mice. Conversely, somatostatin acting on somatostatin receptors on nociceptors impairs the ability of capsaicin to produce behavioral changes and, in an *in vitro* skinnerve preparation, reduces neural activity evoked by capsaicin (Carlton *et al.*, 2004).

c. Phosphorylation and Dephosphorylation. TRPV1 is regulated at several other levels (Cortright and Szallasi, 2004). TRPV1 is directly regulated by phosphorylation through the action of several protein kinases (Fig. 10) and by dephosphorylation by protein phosphatases (Szallasi and Blumberg, 1999). Protein kinases that phosphorylate TRPV1 include PKA and PKC (Premkumar and Ahern, 2000; De Petrocellis et al., 2001; Bhave et al., 2002; Di Marzo et al., 2002; Olah et al., 2002). However, phosphorylation of at least one CaMKII consensus site on TRPV1 is required for capsaicin to evoke a current; apparently, phosphorylation by CaMKII is necessary for the binding of capsaicin to TRPV1 (Jung et al., 2004). By contrast, a capsaicin current can still be elicited despite mutations of the PKA or PKC consensus sites, provided that CaMKII phosphorylation has occurred (Jung et al., 2004). CaMKII has been shown to be localized in many small DRG cells and in terminals in the superficial dorsal horn (Brüggemann et al., 2000). It is found in both small myelinated and unmyelinated axons of peripheral nerves, and its expression is enhanced in response to inflammation (Carlton, 2002). Double-labeling experiments show that 38% of the DRG cells that contain CaMKII colocalize TRPV1: colocalization of other markers of nociceptors, including IB4 binding and the presence of CGRP, was also demonstrated (Carlton and Hargett, 2002). CaMKII could be activated in nociceptive sensory neurons by the influx of Ca^{2+} ions through NMDA receptor channels, TRPV1, or other Ca²⁺ permeable channels. It has been shown that intradermal injection of capsaicin results in an increased expression and an increased phosphorylation of CaMKII in the superficial layers of the dorsal horn, presumably in part within primary afferent terminals (Fig. 11) (Fang, L. et al., 2002a).

The desensitization of TRPV1 is not affected by okadaic acid, a selective inhibitor of protein phosphatases 1 and 2A. However, activation of calcineurin (protein phosphatase 2B) reverses the phosphorylation of TRPV1, and inhibition of calcineurin (by FK-506) reduces the desensitization. Thus, desensitization depends on the dephosphorylation produced during repeated administrations of capsaicin (Docherty *et al.*, 1996; Jung *et al.*, 2004). Desensitization appears to result from Ca²⁺ influx, which causes calcineurin activation and a loss in the ability of ligands to bind to TRPV1 because of the dephosphorylation (Jung *et al.*, 2004).

Phosphorylation results in sensitization of TRPV1, although PKC does not appear to gate the channel directly (Bhave *et al.*, 2003). A number substances can activate PKA and PKC (e.g., bradykinin, ethanol, nicotine, insulin), and so these could help sensitize nociceptors by changing the responsiveness of TRPV1 (Cortright and Szallasi, 2004). Nerve growth factor is also important for the expression of TRPV1 (Chuang *et al.*, 2001).



FIGURE 11 Immunostaining of CaMKII (A–F) and phospho-CaMII (H–M) in the dorsal horn of the lumbar spinal cord of rats following intradermal injection of capsaicin into the foot. The sections in (A–C) were from vehicle-injected animals and in (D–F) from capsaicin injected animals. The sections in (A) and (D) were at 5 min, (B) and (E) at 15 min, and (C) and (F) at 60 min after the injections. Similarly, the sections in (H–J) were from vehicle injected animals and those in (K–M) were from capsaicin injected animals. The sections in (H) and (K) were at 5 min, (I) and (L) at 15 min, and (J) and (M) at 60 min after the injections. The bar graphs at the right show the results of image analysis of the immunostained sections. In (G), CaMKII expression is shown to have increased by 15 min, and in (N) phospho-CaMKII is shown to have increased within 5 min after capsaicin injection. Reprinted from Fang, L. *et al.* (2002a).

d. Subcellular Compartments. Still another level of regulation is by formation of subcellular compartments. It is critical for $[Ca^{2+}]_i$ to be carefully regulated, since excessive activation of TRPV1 by capsaicin or its potent agonist, resiniferatoxin, can desensitize or kill nociceptive neurons or nerve terminals containing TRPV1 because of Ca^{2+} influx (Jeftinija *et al.*, 1992; Nolano *et al.*, 1999; Olah *et al.*, 2001; Neubert *et al.*, 2003). Advantage of this is taken in the treatment of pathological conditions (Section II.C.2.f.).

TRPV1 is found not only in the plasma membrane but also in the endoplasmic reticulum. The TRPV1 in the endoplasmic reticulum helps regulate $[Ca^{2+}]_i$ levels (Cortright and Szallasi, 2004). To activate the TRPV1 receptors in the endoplasmic reticulum, capsaicin or resiniferatoxin can be applied in conjunction with ruthenium red, the latter acting to block TRPV1 located in the plasma membrane. It has also been shown that there is an overlap between TRPV1 in the endoplasmic reticulum and calcium stores that are sensitive to thapsigargin (Karai *et al.*, 2004a).

e. Changes in Gene Expression. Changes in gene expression can also affect TRPV1. For example, there is an upregulation of TRPV1 during inflammation and in experimental neuropathy (Cortright and Szallasi, 2004).

f. Removal of TRPV1-Containing Sensory Neurons. Removal of sensory neurons containing TRPV1 has been used in animals to assess the importance of these receptors in various experimental pain models, including inflammatory hyperalgesia, cancer pain, and arthritic pain (Olah *et al.*, 2001; Karai *et al.*, 2004b). The sensory neurons were ablated, not just desensitized, by the excitotoxicity produced by resinferotoxin injected into multiple sensory ganglia or intrathecally, resulting in the elimination of the pain state without affecting touch, proprioception, mechanical nociception, or locomotor function. Subcutaneous injection of resiniferotoxin reduced thermal nociception for only 20 days, in contrast to the other routes of administration, in which analgesia lasted for at least a year and was presumably permanent. This shorter time course was attributed to killing just nociceptor terminals, rather than eliminating entire sensory neurons.

g. Changes in pH. Lowering the pH is also known to activate TRPV1, causing depolarization and thereby excitation of TRPV1-containing primary afferent neurons (Fig. 10) (Tominaga et al., 1998). Acidic conditions contribute to inflammatory pain (Steen et al., 1992, 1996), and this may in part be due to activation of TRPV1 (Tominaga et al., 1998). However, a separate family of transducer molecules, the ASICs, are also responsive to lowered pH, are present in small primary afferent neurons as well as in CNS neurons, and can activate nociceptors (Bassilana et al., 1997; Waldmann et al., 1997a, b; Waldmann and Lazdunski, 1998; Olson et al., 1998; Habelt et al., 2000; Sutherland et al., 2001; Alvarez de la Rosa et al., 2002). ASICs are upregulated in inflammation as a result of the actions of such inflammatory mediators as NGF, 5HT, interleukin-1, or bradykinin (Mamet et al., 2002).

As already discussed, ASICs belong to the DEG/ENaC family of cation channels (Chen *et al.*, 1998; Waldmann and Lazdunski, 1998; Benson *et al.*, 2002; Xie *et al.*, 2002). In neurons from mice in which the *DRASIC* gene (an acid-sensitive ion channel found exclusively in DRG cells, also called ASIC3) was deleted, there were still H⁺-gated currents, but these had a slowed rate of desensitization, reduced sensitivity to pH, and an enhanced sensitivity to amiloride (Xie *et al.*, 2002). Deletion of the gene for ASIC3 had a dramatic effect on the responses of mice to painful stimuli but in an

unexpected direction (Chen *et al.*, 2002). The knockout mice showed enhanced responses to moderate and intense stimuli, as demonstrated by a decreased latency in the acetic acid writhing test and the hot plate test, and a reduced threshold to tail pressure. It was suggested that ASIC3 helps modulate pain transmission (Chen *et al.*, 2002).

There can be an interaction between temperature and reduced pH (Sugiura *et al.*, 2003) on the responses of small DRG cells from rats. Exposure of the DRG cells to a solution having a low pH often resulted in both transient and sustained depolarizations, although more commonly there was only a sustained response. The transient response was large and was able to trigger action potentials. Transient responses could be blocked by treatment with the ASIC inhibitor, amiloride, whereas this agent failed to affect sustained responses. On the other hand, the TRPV1 receptor antagonist, capsazepine, did not affect the transient response but could partially block the sustained responses when the temperature was in the physiological range or when the pH was lowered further. These experiments suggest that the responses of ASICs to reduced pH are greater at lower temperatures, whereas the responses of TRPV1 to decreased pH are greater at higher temperatures.

D. Itch Sensation

Another class of response to a chemical stimulus is the sensation of itch. This sensation can occasionally be produced during intraneural microstimulation of C-fibers in human subjects (Torebjörk and Ochoa, 1983). It has now been shown that stimulation using iontophoretic application of histamine to human skin produces an itch sensation and activates a distinctive set of mechanically insensitive C-fibers (Schmelz *et al.*, 1997). Some of these were also shown to respond to heat and some to capsaicin, and they had very large receptive fields. Presumably, itch transduction depends on the activation of histamine receptors on this specific group of sensory neurons.

III. SENSITIZATION OF NOCICEPTORS

Nociceptors can be sensitized following strong noxious stimuli or by exposure to inflammatory mediators or low pH (Bessou and Perl, 1969; Fitzgerald and Lynn, 1977; Meyer and Campbell, 1981; LaMotte *et al.*, 1982, 1983; Kocher *et al.*, 1987; Reeh *et al.*, 1987; Häbler *et al.*, 1990; Kirchhoff *et al.*, 1990; Handwerker *et al.*, 1991; Schmelz *et al.*, 1996; Willis and Coggeshall, 2004).

The following substances can induce sensitization of nociceptors by interactions with surface membrane receptors: **bradykinin** (Rueff and Dray, 1993a; Schuligoi *et al.*, 1994; Liang *et al.*, 2001; Shin *et al.*, 2002); **catecholamines** (Gold *et al.*, 1994; Khasar *et al.*, 1999); **cytokines** (Sorkin *et al.*, 1997); **excitatory amino acids** (Ferreira and Lorenzetti, 1994; Du *et al.*, 2001); **inflammatory soup** (a mixture of bradykinin, histamine, serotonin, and prostaglandin E_1) (Kessler *et al.*, 1992; Davis, *et al.*, 1993; Kress *et al.*, 1997); **neurokinins** and other **peptides** (Nakamura-Craig and Gill, 1991; Kessler *et al.*, 1992); **prostaglandins** and other arachidonic acid products (Martin *et al.*, 1987; Cohen and Perl, 1990; Martin, 1990; White *et al.*, 1990; Schepelmann *et al.*, 1992; Rueff and Dray, 1993a,b; Chen *et al.*, 1999); **protons** (Steen *et al.*, 1992, 1996); and **serotonin** (Rueff and Dray, 1993b).

A. Growth Factors

Growth factors, such as NGF, also contribute to the sensitization of nociceptors during inflammation (Koltzenburg *et al.*, 1999). Nerve growth factor can cause sensitization of $A\delta$ nociceptors to mechanical stimuli when administered during the neonatal period (Lewin *et al.*, 1993) and to heat stimuli in adult animals (Lewin *et al.*, 1993). This appears to correlate with changes in somal membrane properties. For instance, chronic NGF treatment resulted in broadened action potentials in nociceptive DRG cells (Ritter and Mendell, 1992). Animals chronically treated with NGF from birth to adulthood develop a profound mechanical hyperalgesia but not mechanical allodynia (Lewin *et al.*, 1993). Administration of NGF to adult animals rapidly produces heat hyperalgesia, probably by release of substances that sensitize heat nociceptors.

B. Noxious Stimuli

The noxious stimuli that cause sensitization can be mechanical, thermal, or chemical. Depending on the circumstances and the mechanisms engaged, sensitization can be selective for mechanical, thermal, or chemical stimuli. Low pH sensitizes the responses of nociceptors to mechanical stimuli (Steen *et al.*, 1992) and inflammatory agents (Steen *et al.*, 1995b, 1996). Kress *et al.* (1997) found that capsaicin-responsive DRG cells cultured in the presence of NGF show an increased inward current when exposed to inflammatory mediators at acidic but not at neutral pH. Low pH also increases the responses of small DRG cells to capsaicin (Vyklický *et al.*, 1998). Capsazapine, which was developed as a selective antagonist of TRPV1 receptors

(Bevan *et al.*, 1992), blocked the increase in responses produced by inflammatory mediators at low pH, although it did not affect currents evoked by low pH itself, indicating that the changed responses involved the capsaicin receptor (Vyklický *et al.*, 1998). The independent actions of low pH and capsazepine may indicate different recognition sites on the TRPV1 receptors or may reflect an action of protons that is mediated by ASICs. The responses of nociceptors to capsaicin can also be sensitized by proinflammatory cytokines such as tumor necrosis factor- α (Nicol *et al.*, 1997a). The effect is mediated by prostaglandin production.

Sensitization can be restricted to just a part of the terminal arborization of a nociceptor (Schmelz *et al.*, 1996). This indicates that the intracellular mechanisms of sensitization are localized to the part of a nociceptive axon that is affected by the sensitizing agent.

C. Signal Transduction Pathways

The sensitization of nociceptors depends on the activation of signal transduction pathways in the nociceptive sensory neurons. For example, PGE₂ increases the responses of DRG cells to K⁺ ions (Baccaglini and Hogan, 1983) and bradykinin (Cui and Nicol, 1995). This effect is mediated by activation of the cAMP signaling pathway, since PGE₂ produces increased cAMP levels in sensory neurons (Hingtgen et al., 1995), the administration of membrane-permeant cAMP analogs sensitize DRG cells, and PKA inhibitors block PGE₂ sensitization (Cui and Nicol, 1995). The cAMP cascade mediates the enhancement of capsaicin-evoked currents in rat DRG cells by activation of PKA, as shown by the similarity in currents evoked by PGE₂, forskolin, but not D-forskolin, and 8-bromo-cAMP, and by the ability of a PKA inhibitor to prevent the effects of PGE₂ or forskolin (Lopshire and Nicol, 1998). Capsaicin activated single channels (cf., Oh et al., 1996), and this action was blocked by capsazepine. PGE₂ or forskolin administration increased the number of active channels and/or increased the channel opening probability; however, single channel conductance was unaffected. On the other hand, activation of PKG has been found to prevent the PGE₂ sensitization of the capsaicin-evoked current (Lopshire and Nicol, 1997).

Repetitive activation of DRG neurons by electrical stimulation (to imitate the effects of injury) resulted in an increase in the fast currents evoked by ATP (Xu and Huang, 2004). This effect was blocked by KN93, an inhibitor of CaMKII and not by the inactive KN92. The increase in ATP currents was also blocked by removal of Ca^{2+} from the bathing medium or by blocking calmodulin. The DRG cells were shown to upregulate P2X₃ receptors as well as CaMKII and phosphorylated CaMKII. The increase in P2X₃ receptors

included those associated with the surface membranes of the neurons, indicating increased trafficking of these receptors. It was suggested that injury could produce peripheral sensitization by this CaMKII-mediated upregulation of $P2X_3$ receptors.

IV. ROLE OF VOLTAGE-GATED SODIUM AND POTASSIUM CHANNELS IN PERIPHERAL SENSITIZATION

A. Voltage-Gated Sodium Channels

Sensitization of nociceptors is related not only to alterations in transducer molecules, such as TRPV1, but also in membrane currents through voltagegated sodium channels (Lai et al., 2004). There are a number of voltage-gated sodium channels of variable properties and tissue location. The variations relate to the subunit composition, which includes an α subunit and one or more β subunits. There are 10 types of α subunit and 3 types of β subunit. The α subunit forms the functional ion channel, including the pore, voltage sensor, selectivity filter, fast inactivation site, the binding site for tetrodotoxin, and several sites, at which phosphorylation can occur to modulate gating properties. β subunits assist in targeting and anchoring the channels and modulation of the properties of the α subunit. Different combinations of subunits underlie the subdivision of sodium channels into nine classes named $Na_v 1.1$ to $Na_v 1.9$. Various types of sodium channels may be differentially expressed in different types of sensory neurons. For example, Na_v1.8 (formerly called PN3 or SNS) is found mainly in small neurons, although it is also in some medium- and large-sized neurons (Djouhri et al., 2003), whereas Nav1.9 is found only in nociceptive sensory neurons (Fang, X. et al., 2002b). Essentially all sensory ganglion cells contain Nav1.6 and Nav1.7. These are respectively localized chiefly in nodes of Ranvier and in axon endings. On the other hand, Nav1.8 is located chiefly in the cell body, and Nav1.9 is found throughout neurons with unmyelinated axons.

Voltage-gated sodium channels may be either sensitive or resistant to the blocking effect of tetrodotoxin (TTX). Of particular interest are tetrodotoxinresistant sodium channels that are specifically located in sensory neurons (Sangameswaren *et al.*, 1996; Akopian *et al.*, 1999). There are three types of TTX-resistant sodium currents (Lai *et al.*, 2004). One has a low threshold for activation, with rapid rates of activation and inactivation. This current may use Na_v1.5. Another TTX-resistant sodium current has a high threshold for activation; it activates and inactivates slowly. These properties resemble those seen in nociceptors. The current may depend on Na_v1.8. A third type of TTX-resistant sodium current has a low threshold for activation, but the channel is available over a wide range of potentials, with a midpoint at -44 mV. The current is considered persistent because it has a very slow rate of activation. It probably does not trigger action potentials, but rather it helps determine the membrane potential and is responsible for subthreshold depolarizations. This current appears to depend on Na_v1.9.

Transgenic mice lacking the gene for $Na_v 1.8$ (or SNS) appear to be normal, but they are analgesic to noxious mechanical stimuli applied to the tail. They have reduced responses to noxious thermal stimuli in some tests, and the development of inflammatory hyperalgesia following an intraplantar injection of carrageenan is delayed (Akopian *et al.*, 1999). Other evidence that $Na_v 1.8$ has a nociceptive function is the reversal of neuropathic pain in rats treated with antisense oligonucleotides that affect expression of this channel (Lai *et al.*, 2002).

The action potentials of nociceptors have a distinct hump on the downslope (Ritter and Mendell, 1992). This reflects the influx of Ca^{2+} ions during the repolarization phase of the action potential (Blair and Bean, 2002). This hump may in part be due to the presence of TTX-resistant sodium channels in nociceptors (Lai *et al.*, 2004). The influx of Ca^{2+} ions would be an important step in the process of sensitization of nociceptors by activation of intracellular signaling pathways.

Exposure of nociceptors to the prostaglandin, PGE_2 , facilitates the sodium current that enters the nociceptors through TTX-resistant sodium channels (England *et al.*, 1996; Gold *et al.*, 1996b, 1998; Khasar *et al.*, 1998) and reduces the outward current through a potassium channel (Nicol *et al.*, 1997b). As in the case of sensitization of TRPV1 receptors, these effects on voltage-gated sodium and potassium channels are mediated through activation of the cAMP signaling cascade (Lopshire and Nicol, 1998; Fitzgerald *et al.*, 1999). The TTX-resistant sodium current can also be increased by activation of the PKC signaling pathway.

The sensitization of nociceptors by other inflammatory mediators is also often through the modulation of TTX-resistant sodium currents, and this can lead to primary hyperalgesia (Lai *et al.*, 2004).

B. Voltage-Gated Potassium Channels

Sensory neurons also contain a variety of voltage-gated potassium channels. At least six different potassium currents could be distinguished by Gold *et al.* (1996a) in DRG neurons. Three of these were transient (I_{Af} , I_{Aht} , and I_{As}), inactivating during maintained depolarization; the other three did not inactivate (I_{Ki} , I_{Klt} , and I_{Kn}). Only one of these was dominant in a given

DRG neuron. DRG cells were divided into those considered to be nociceptors, based their small size, sensitivity to capsaicin, and the presence of a shoulder on the action potential, and those considered not to be nociceptors. I_{Aht} is preferentially expressed in presumed nociceptors, as are I_{As} and I_{Ki} . I_{Af} is selectively expressed in nonnociceptors, and I_{Klt} and I_{Kn} are found in both sets of DRG cells. An issue is which of these potassium channels are transported to and inserted into the sensory nerve terminal membranes and thus affect afferent excitability.

Rasband *et al.* (2001) use a different terminology for their list of voltagegated potassium channels. Myelinated axons contain chiefly $K_v1.1$, $K_v1.2$, and $K_v\beta2.1$ channels. Unmyelinated axons have $K_v1.1$ and/or $K_v1.2$ assembled with $K_v1.4$. Nerve injury resulted in a downregulation of K_v channels. This should enhance excitability, helping account for the development of ectopic discharges in animals with experimental neuropathic pain.

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CHAPTER 4

TRPV1: A Polymodal Sensor in the Nociceptor Terminal

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I. DISCOVERY AND CLONING OF THE VANILLOID RECEPTOR

A. Study of Pungent Peppers Leads to Characterization of a "Vanilloid" Receptor

"Hot" peppers have been used in food and ritual for millenia. However, the scientific basis of their sensory effects remained a mystery until 1846, when Thresh purified the main pungent ingredient of capsicum peppers, capsaicin (Thresh, 1846). In 1928, the chemical structure of capsaicin was solved by Nelson as 8-methyl-N-vanillyl-6-noneamide (Nelson, 1919) (Fig. 1). Several decades later, work performed largely by Jancso, Szolcanyi, and colleagues in Hungary revealed that capsaicin could both cause pain and desensitize rats to further capsaicin challenge. Moreover, they demonstrated that high-dose capsaicin administration to neonatal rats could trigger the irreversible degeneration of a specific subset of thin, unmyelinated afferent neurons responsible for pain sensation (Jancso et al., 1967, 1977; Szolcsanyi and Jancso-Gabor, 1975, 1976; Szolcsanyi, 1977). In the 1980s, Blumberg and colleagues came to the critical realization that capsaicin and resiniferatoxin (RTX) (Fig. 1), a pungent phorbol ester derived from Euphorbia species, share similar chemical structures, including a vanilloid motif that is essential for pungency (deVries and Blumberg, 1989; Szallasi and Blumberg, 1989). These investigators also provided evidence for specific binding of [³H] RTX (and its displacement by capsaicin) to the cell bodies and spinal projections of small-diameter primary sensory neurons located in the dorsal root and trigeminal ganglia (Szallasi and Blumberg, 1990). The subsequent development of an antagonist of vanilloid action, capsazepine (Fig. 1), by Bevan and colleagues allowed the "vanilloid receptor" to be further defined as a distinct pharmacological site (Bevan et al., 1992).

Data derived from the application of electrophysiological and fluorescent calcium imaging methods to cultured sensory neurons or explanted ganglia provided further evidence that vanilloid receptor binding could lead to the activation of nociceptive neurons by triggering the activation of a nonselective cation channel, leading to Na⁺ and Ca²⁺ influx (Williams and Zieglgansberger, 1982; Baccaglini and Hogan, 1983; Heyman and Rang, 1985; Marsh *et al.*, 1987; Winter, 1987; Bleakman *et al.*, 1990; Bevan and Docherty, 1993; Oh *et al.*, 1996). These events have two consequences. First, they trigger action potential generation and the activation of ascending nociceptive signaling pathways that lead to the perception of pain. Second, in a subset of nociceptive neurons, they result in the so-called axon reflex response in which the proinflammatory neuropeptides substance P and calcitonin gene-related peptide are released from vesicles in all of the peripheral branches of the activated nociceptor terminal to cause neurogenic



TRPV1 agonists

FIGURE 1 Representative chemical agonists and antagonists of endogenous and recombinant TRPV1.

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FIGURE 2 TRPV1 is a nonselective cation channel that can be activated by vanilloid compounds, protons, or heat. (A) Representative current traces obtained from voltage-clamped Xenopus laevis oocytes transfected with recombinant TRPV1. Holding potential is -40 mV. Cells were stimulated with capsaicin (cap), protons, or an elevation in bath temperature, as indicated. (B) Response of TRPV1, expressed in Xenopus laevis oocytes, to acute temperature fluctuations. (C) Temperature-response profile of voltage-clamped HEK293 human embryonic kidney cells transiently transfected with TRPV1 (open squares) or control vector (filled circles) at -60 mV. (D) Reversible single-channel responses of excised inside-out membrane patches

inflammation (Szolcsanyi, 1984b). For a more extensive treatment of these issues, the reader is referred to several authoritative reviews (Szolcsanyi, 1984a; Holzer, 1991; Wood, 1993; Szallasi, 1994).

B. Molecular Cloning of a Vanilloid Receptor, TRPV1

In 1997, using an expression-cloning strategy based on the known properties of the vanilloid receptor, Julius and colleagues isolated a single cDNA from rat sensory ganglia that, on transfection into nonneuronal cells, resulted in the appearance of a capsaicin- and RTX-gated nonselective cation channel (Caterina et al., 1997) (Fig. 2). The 838 amino acid protein encoded by this cDNA was correspondingly named vanilloid receptor 1 (VR1). Consistent with its functional properties, VR1 was predicted to exhibit an ion channel-like structure, consisting of six transmembrane domains (S1-S6), a pore-loop domain between S5 and S6, and cytosolic amino and carboxyl termini (Fig. 3). The amino terminus was found to contain three ankyrin repeat domains, 33 amino acid motifs that appear in numerous membrane and soluble proteins, and that often participate in protein-protein interactions. Using a more current definition of the ankyrin repeat consensus, a fourth such domain is observed closer to the amino terminus. VR1 turned out to be a member of the transient receptor potential (TRP) family of ion channel proteins, now known to contain at least 30 members divided into 7 subfamilies (TRPC, TRPV, TRPM, TRPP, TRPN, TRPA, and mucolipin) (Montell et al., 2002a; Clapham, 2003). In an effort to streamline TRP channel nomenclature, VR1 was subsequently renamed TRPV1, the first member of the TRPV (vanilloid) subfamily (Montell et al., 2002b).

Studies employing native gel electrophoresis or the coexpression of wild-type and "dominant negative" mutant TRPV1 have provided evidence that TRPV1 exists as a tetramer in the plasma membrane (Kedei *et al.*, 2001;

derived from TRPV1-expressing HEK293 cells to bath-applied capsaicin (100 nM, left) or heat (right). Traces from control vector-transfected cells are at top. Reversal potential = +40 mV. Channel inhibition by capsazepine (czp, 10 μ M) is also illustrated. (E) Current-voltage relationship illustrating outward rectification of single-channel TRPV1-mediated responses to heat. (F) *In situ* hybridization analysis of TRPV1 expression in rat DRG, indicating selective expression in small-diameter neurons (arrow) and lack of expression in large-diameter neurons (arrowhead). Panels B, D, and E reprinted from Tominaga *et al.*, *Neuron* **21**, 531–543, copyright 1998, with permission from Elsevier. Panels A, C, and F reprinted from Caterina *et al.*, *Nature* **389**, 816–824, copyright 1997 and Caterina *et al.* Nature **398**, 436–441, copyright 1999, with permission from Nature Publishing Group.



FIGURE 3 Topological model of TRPV1 and illustration of domains critical for function. See text for abbreviations.

Kuzhikandathil *et al.*, 2001; Garcia-Sanz *et al.*, 2004), as previously demonstrated for voltage-gated K⁺ channels and cyclic nucleotide-gated channels. TRPV1 tetramerization appears to depend on a sequence in the proximal carboxyl terminus, the TRP domain, which is conserved among TRP family members (Garcia-Sanz *et al.*, 2004) (Fig. 3). A peptide derived from this region, extending from Glu 648 to Arg 721, forms stable multimers *in vitro*. Moreover, deletion of this sequence from TRPV1 disrupts multimeric channel assembly. It has also been demonstrated that TRPV1 undergoes N-linked glycosylation, most likely at Asn 604 (Jahnel *et al.*, 2001).

On exposure to capsaicin, TRPV1 exhibits an outwardly rectifying, nonselective cationic current (Fig. 2). Studies have demonstrated that this channel also exhibits a certain degree of voltage-dependent gating (Gunthorpe *et al.*, 2000; Voets *et al.*, 2004) such that depolarization promotes TRPV1 activation, whereas hyperpolarization promotes channel closing. Like many members of the TRP family, TRPV1 distinguishes poorly among monovalent cations but exhibits a five- and tenfold higher relative permeability to Mg^{2+} and Ca^{2+} , respectively (Caterina *et al.*, 1997). However, the open TRPV1 pore appears to be unusually wide, since large cations, such as Co^{2+} , H_3O^+ , and even the organic dye FM1–43 can permeate the activated

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channel (Winter, 1987; Meyers *et al.*, 2003; Hellwig *et al.*, 2004). Capsaicin evokes the opening of discrete single channels (\sim 80 pS at positive potentials, \sim 35 pS at negative potentials) in cell-attached, inside-out and outside-out membrane patches from cells expressing TRPV1, and these openings exhibit multiple subconductance states (Caterina *et al.*, 1997; Nagy and Rang, 1999b; Premkumar *et al.*, 2002). At both the whole-cell and single channel levels, the functional properties of recombinant TRPV1 largely recapitulate those of endogenous capsaicin receptors in sensory neurons.

Several laboratories have demonstrated that in addition to mediating the flux of cations across the plasma membrane, TRPV1 can also trigger the release of Ca^{2+} ions from intracellular stores in both transfected HEK cells and sensory neurons (Marshall *et al.*, 2003; Liu *et al.*, 2003b; Karai *et al.*, 2004b). While some discrepancies exist regarding the details of these responses, it appears that TRPV1 mediates release from both IP₃-sensitive and IP₃-insensitive stores. In addition, whereas capsaicin-evoked Ca^{2+} release appears to be mediated through cell-surface TRPV1, Ca^{2+} release in response to RTX may also involve TRPV1 channels located intracellularly.

II. TRPV1 EXHIBITS A HIGHLY SPECIFIC EXPRESSION PATTERN

Localization studies, involving *in situ* hybridization, immunohistochemistry, immunoblot, and the reverse transcriptase-polymerase chain reaction, have revealed that TRPV1 is highly expressed in dorsal root, trigeminal, and nodose ganglia, specifically within a subset of small- to medium-diameter sensory neurons that project to the spinal cord (laminae I and II), trigeminal nucleus, and solitary tract nucleus, respectively (Caterina *et al.*, 1997; Tominaga *et al.*, 1998; Guo *et al.*, 1999; Michael and Priestly, 1999; Ma, 2002). This pattern is completely consistent with the selectivity of capsaicin action at thin unmyelinated C-fibers and lightly myelinated $A\delta$ -fibers (Holzer, 1991). Although TRPV1 expression is also observed in a number of other neuronal and nonneuronal locations, including many areas of the brain and epithelial cells of the skin and urinary bladder (Sasamura *et al.*, 1998; Mezey *et al.*, 2000; Schumacher *et al.*, 2000; Birder *et al.*, 2001; Denda *et al.*, 2001), the expression level of TRPV1 in sensory ganglia appears to be at least 30-fold greater than in any of these other regions (Sanchez *et al.*, 2001).

Even among sensory neurons, TRPV1-expressing cells are histologically heterogeneous. In the rat, where substance P expression and binding to the isolectin B4 represent markers for two largely nonoverlapping C-fiber sub-populations (Snider and MaMahon, 1998), TRPV1 immunoreactivity is observed in ~85% of substance P-positive neurons, 60-80% of IB4 positive neurons, and ~40% of neurons overall (Tominaga *et al.*, 1998; Guo *et al.*,

1999). In the mouse, strong TRPV1 immunoreactivity is also seen in many substance P-positive neurons but is more difficult to detect in IB4-positive neurons (Woodbury *et al.*, 2004). In contrast, physiological studies of wild-type and TRPV1 knockout mice (Section V.C.) strongly support the expression of TRPV1 in this IB4 positive population (Caterina *et al.*, 2000). The reason for this discrepancy is unclear but may reflect epitope masking or the expression of TRPV1 splice variants within these cells.

Orthologs of TRPV1 have been isolated from many species, including rat (Caterina *et al.*, 1997), human (Hayes *et al.*, 2000; McIntyre *et al.*, 2001), mouse (Chung *et al.*, 2003; Wang *et al.*, 2004), chicken (Jordt and Julius, 2002), rabbit (Gavva *et al.*, 2004), and guinea pig (Savidge *et al.*, 2002). As predicted from behavioral, electrophysiological, and RTX binding studies, however, only the mammalian orthologs exhibit capsaicin sensitivity.

Several TRPV1 splice variants have also been identified. One of these is VR.5'sv, which is lacking much of the amino terminus found in the fulllength form of TRPV1 (Schumacher *et al.*, 2000). Another splice variant, TRPV1b, was isolated from mouse and is characterized by a 10 amino acid deletion in the amino terminus, within the third ankyrin repeat domain (Wang *et al.*, 2004). Neither splice variant, when expressed alone, exhibits responses to capsaicin or any other TRPV1 agonist. However, equimolar coexpression of TRPV1b with the full-length mouse TRPV1 (TRPV1a) reduces agonist-evoked activity of the full-length form. The physiological significance of the latter finding remains to be established, however, since the TRPV1b isoform is typically expressed at a lower level than that of full-length TRPV1. The relative expression levels of these splice variants following inflammation or other pathological insults also have yet to be determined.

III. ACTIVATORS AND INHIBITORS OF TRPV1

A. Diverse Chemical Activators of TRPV1

Vanilloid activators of TRPV1 include not only capsaicin (EC₅₀ ~16– 111 nM) (Tominaga *et al.*, 1998; McIntyre *et al.*, 2001; Gavva *et al.*, 2004) and RTX (EC50 ~3 nM) (Szallasi *et al.*, 1999; Gavva *et al.*, 2004) but also zingerone (EC₅₀ ~75 μ M) and piperine (EC₅₀ ~3 μ M), chemicals responsible for the pungency of ginger and black pepper, respectively (Sterner and Szallasi, 1999; Witte *et al.*, 2002). In addition to these vanilloid compounds and their structural relatives, ethanol (1–3%) (Trevisani *et al.*, 2002), reducing agents (Vyklicky *et al.*, 2002) and certain plant-derived unsaturated 1,4-dialdehydes (e.g., isovelleral, Fig. 1) (Sterner and Szallasi, 1999) are also capable of activating or potentiating TRPV1.

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Despite the importance of these various exogenous substances in the study and manipulation of TRPV1, however, none of them are typically produced in mammals. What, then, might be the "physiological" regulator of vanilloid receptors? It turns out that TRPV1 can be activated by a diverse host of chemical and physical stimuli normally encountered *in vivo*. Protons, for example, can both stimulate TRPV1 (half-maximal activation at pH 5.4, Fig. 2) and sensitize it to capsaicin or other agonist (Caterina *et al.*, 1997; Tominaga *et al.*, 1998). As described in Section III.D., these sensitization and activation phenomena are experimentally separable. Protons are released at high concentrations in inflamed, ischemic, or neoplastic tissues, where the pH can drop to as low as 5 (Bevan and Geppetti, 1994). It has therefore been proposed that proton actions on TRPV1 underlie some aspects of ischemic or inflammatory pain and hyperalgesia.

Numerous endogenous lipid metabolites of arachidonic acid or other fatty acids are also capable of activating TRPV1. One of these is 12 HPETE, a lipoxygenase product of arachidonic acid (Hwang et al., 2000). A very interesting group of fatty acid-derived TRPV1 agonists consists of the socalled endocannabinoids (Van Der Stelt and Di Marzo, 2004). These fatty acyl amides include the cannabinoid receptor agonist, palmitoyl ethanolamide (anandamide), as well as several discovered amide lipids such as N-arachidonyl dopamine (NADA) (Zygmunt et al., 1999; Huang et al., 2002) (Fig. 1). One feature that makes these compounds particularly compelling as candidate endogenous TRPV1 modulators is their widespread distribution, not only in peripheral tissues innervated by TRPV1-expressing afferents but also in the spinal cord and brain. The latter finding may help to explain why TRPV1 is expressed throughout the central nervous system (CNS), albeit at levels lower than in nociceptive neurons. For example, the substantia nigra pars compacta, which contains presynaptic TRPV1-expressing terminals, also contain significant quantities of anandamide, which may account for the "vanilloidergic" tone recorded in slice preparations of this region (Marinelli et al., 2003). The chemical structure of fatty acyl amides has guided the development of additional synthetic TRPV1 agonists such as the ultrapotent vanillamide phenylacetylrinvanil (EC50 = 11 pM) (Appendino et al., 2004).

Another interesting finding is that some fatty acyl amides, such as *N*-palmitoyl dopamine, are not capable of activating TRPV1 alone but when coadministered with bona fide TRPV1 agonists, like anandamide, contribute to a so-called entourage effect, enhancing the potency or efficacy of the co-applied agonist (De Petrocellis *et al.*, 2004). The molecular basis for this effect is not entirely clear, but it may relate to cooperativity between binding sites on subunits within the functional tetramer. Indeed, concentration-response analysis indicates a Hill coefficient of approximately 2 for capsaicin

or RTX binding to and activation of TRPV1 (Caterina *et al.*, 1997; Szallasi *et al.*, 1999). Together with the potentiation of TRPV1 by protons, TRPV1 activation by heat (Fig. 2, Section III.C.) and TRPV1 modulation by phospholipids binding and phosphorylation (see later), the entourage effect of endocannabinoids highlights the ability of TRPV1 to integrate diverse environmental stimuli. *In vivo*, such capacity for integration might make possible the exquisite fine-tuning of this channel's activity under various physiological and pathological conditions.

An additional source of complexity, with regards to TRPV1 agonists, is that they exhibit variable relative abilities to activate TRPV1, desensitize TRPV1, desensitize nociceptor terminals, or evoke nocifensive behaviors. Resiniferatoxin, for example, produces a sensation of pungency, like capsaicin, but is relatively more effective at desensitizing vanilloid-sensitive neurons (Szolcsanyi *et al.*, 1990; Acs *et al.*, 1997). Olvanil (Brand *et al.*, 1990) and capsiate (Iida *et al.*, 2003), while capable of activating TRPV1 *in vitro*, are nonpungent. These differential properties likely originate in differential hydrophobicities, on and off rates for TRPV1 binding, and stability *in vivo*. Another possibility that cannot be excluded, however, is that they reflect the induction of distinct active conformations of TRPV1.

B. Chemical Antagonists of TRPV1

The two chemical agents that have been used traditionally to block capsaicin receptors are capsazepine and ruthenium red. Capsazepine is a thiourea that bears some structural resemblance to vanilloid compounds (Bevan et al., 1992) (Fig. 1). In vitro, this compound competitively antagonizes both vanilloid activation of TRPV1 currents and RTX binding to membranes containing native or recombinant TRPV1. Capsazepine can also block the activation of TRPV1 by protons, anandamide, or heat (Section III.C.). However, there are significant species differences in the potency of capsazepine at blocking responses evoked by nonvanilloid stimuli. For example, whereas protonevoked currents through human or guinea pig TRPV1 can be readily blocked by 1 µM capsazepine, at least tenfold more capsazepine is required to inhibit proton activation of rat TRPV1 (McIntyre et al., 2001). Furthermore, at micromolar concentrations, capsazepine can act on other targets, including nicotinic acetylcholine receptors (Liu and Simon, 1997) and hyperpolarization-activated cation channels (Ray et al., 2003). These factors limit the information that can be obtained from the *in vivo* use of capsazepine. Ruthenium red is a highly charged organic cation that acts as a noncompetitive capsaicin receptor antagonist, apparently by blocking the channel pore (Dray et al., 1990; Maggi, 1993; Caterina et al., 1997). This compound, however, is even more promiscuous than capsazepine, as it blocks a number of nonselective cation channels, binds cell-surface carbohydrates, and inhibits intracellular calcium channels.

Since the cloning of TRPV1, many additional antagonists have been developed that exhibit somewhat better selectivity for this channel. Most of these are halogenated vanilloids [e.g., 5'-iodinated resiniferatoxin (I-RTX)] (Wahl *et al.*, 2001) or compounds based on a urea or amide structure (e.g., SB366791) (Gunthorpe *et al.*, 2004) (Fig. 1). These two particular examples, while useful *in vitro*, have not been employed with much success *in vivo*, perhaps owing to problems with solubility or stability. However, newer TRPV1 antagonists, identified largely through high throughput screening efforts, may prove more suitable for *in vivo* use (Rami and Gunthorpe, 2004).

In addition to these synthetic antagonists, there is evidence that certain endogenously occurring small molecules have the capacity to inhibit TRPV1 activity. One of these is phosphatidyl inositol bisphosphate (PIP₂) (Chuang *et al.*, 2001; Prescott and Julius, 2003). This membrane phospholipid appears to be constitutively associated with TRPV1, suppressing channel gating. On cleavage by phospholipase C or experimental sequestration with PIP₂specific antibodies, however, this lipid is removed from TRPV1, resulting in channel sensitization. It was determined that adenosine can also inhibit TRPV1 activation and competitively block RTX binding, presumably by direct interaction with the channel (Puntambekar *et al.*, 2004). However, TRPV1 inhibition by endogenous adenosine has yet to be demonstrated.

C. TRPV1 is the First Heat-Gated Ion Channel to be Identified

Perhaps the most intriguing feature of TRPV1 is that, in addition to its responsiveness to diverse chemical stimuli, this channel appears to be gated directly by noxious heat. Prior to the cloning of TRPV1, the McNaughton and Levine laboratories had independently demonstrated that a subpopulation of presumably nociceptive rat dorsal root ganglion (DRG) neurons in culture exhibit a nonselective cation current that could be activated simply by increasing the ambient temperature into the noxious range (i.e., >42°C) (Cesare and McNaughton, 1996; Reichling and Levine, 1997). Subsequently, an almost identical heat-evoked current was observed in nonneuronal mammalian or amphibian cells transfected heterologously with recombinant TRPV1 (Caterina *et al.*, 1997) (Fig. 2). This current, like that evoked by vanilloid compounds or protons, was inhibited by capsazepine or ruthenium red. Above the apparent threshold for thermal activation, TRPV1-mediated currents tracked perfectly with fluctuating ambient temperature, consistent

with this molecule acting as a microscopic temperature sensor. Corroboration of these findings has come from the observation that, at the whole-cell level, there is a strong correlation between capsaicin responsiveness and heat responsiveness among sensory neurons (Kirschstein *et al.*, 1997). For reasons that remain unclear, however, this correlation is much weaker at the level of the excised membrane patch (Nagy and Rang, 1999b).

How might heat influence TRPV1 channel opening? Although some degree of thermal sensitivity is observed among nearly all proteins, what sets TRPV1 (and its five mammalian temperature-gated homologs) apart is the steep temperature dependence of its activation. Whereas the Q_{10} for most biochemical processes is <2, the Q_{10} for TRPV1-mediated currents at -60 mV between 42°C and 50°C is closer to 20 (Benham *et al.*, 2003). Moreover, heat-evoked activation of TRPV1 can be observed not only in intact cells but also in membrane patches excised from these cells (Tominaga *et al.*, 1998) (Fig. 2). This latter finding argues against the involvement of a soluble second messenger in the thermal activation of TRPV1 and in favor of a membrane-delimited process. One interpretation of these findings, but one that has yet to be formally proven, is that the TRPV1 protein is itself heat sensitive. At the single channel level, heat dramatically increases the TRPV1 open probability, with a relatively smaller effect on unitary conductance (Tominaga *et al.*, 1998; Liu *et al.*, 2003a).

The precise mechanistic basis for TRPV1 thermal sensitivity remains unclear. However, it was reported by Nilius and colleagues that at least part of this effect might be explained on the basis of the thermodynamics of voltage gating (Voets et al., 2004). These investigators used a classical "tail-current" protocol to examine TRPV1 currents over a broad range of temperatures and voltages. In doing so, they discovered that at 25°C some basal level of TRPV1 activity could be observed, even in the absence of chemical agonists, provided that the TRPV1-expressing cell was clamped at extremely depolarized potentials (>100 mV). As the temperature was progressively increased, there was a continuous increase in the proportion of open TRPV1 channels, as a function of voltage (i.e., the half-maximal voltage for channel opening became progressively less positive). A similar shift in voltage-dependent gating could be observed in response to the administration of capsaicin at room temperature, suggesting a common mechanism for chemical and thermal TRPV1 activation. Analogous behavior was observed in a distantly related thermosensitive ion channel, the could- and menthol-gated ion channel TRPM8. Through mathematical modeling, these investigators arrived at a single thermodynamic model that cold explain the thermal responsiveness of both channels. According to this model, if there exists a significant difference in the effects of temperature on the energies of activation for voltage-gated channel opening versus channel
closing, that channel will exhibit gating by either heat or cold, depending on which activation energy is greater. If the activation energy for channel opening is greater than that of channel closing, the channel will be heatgated. If the activation energy for channel opening is less than that for channel closing, the channel will be cold-gated. This model is consistent with the observation that for most voltage-gated ion channels, the activation energies for opening and closing are more similar than they are for TRPM8 and TRPV1. Another of the authors' conclusion, based on these findings, was that, within the entire range of temperatures tested in this study (18-45°C), increasing temperature results in a continuous enhancement of TRPV1 open probability. At first glance, this observation may seem to be at odds with the observation, described previously, that cells expressing TRPV1 exhibit an apparent threshold in heat-evoked inward current at approximately 42°C. One possible explanation for this apparent discrepancy is that at a given negative holding potential (the conditions used in most nociceptor or TRPV1 recordings), the nonlinear Boltzmann function for voltage-dependent channel opening results in a dramatic enhancement of current over a relatively narrow temperature range. These results notwithstanding, additional temperature-dependent effects on TRPV1 gating cannot be excluded, particularly at temperatures $>40^{\circ}$ C in which the behavior of TRPV1 was not extensively tested.

D. Structure-Function Relationships for Agonist/Antagonist Interaction with TRPV1

The amazing polymodal sensitivity of TRPV1 raises obvious questions regarding the structural mechanisms by which diverse chemical and physical stimuli can regulate this channel's activity. A lack of crystallographic data for any TRP channel has hampered progress in this area. However, mutagenesis studies have begun to provide insight into nature of the interactions between TRPV1 and some of its ligands (Fig. 3). One feature that has been exploited quite effectively, toward this end, is the species variation that exists in TRPV1 responsiveness to different agonists and antagonists.

A prime example of this variation is illustrated by the long-recognized fact that only mammalian nociceptors are sensitive to pungent vanilloid compounds (Szallasi, 1994). Correspondingly, whereas all known mammalian TRPV1 orthologs can be activated by the vanilloid compounds, capsaicin and RTX, nonmammalian TRPV1 orthologs all appear to be heat- and/or proton-sensitive but vanilloid-insensitive. Jordt and Julius took advantage of this phenomenon by constructing chimeric rat-chicken TRPV1 molecules and evaluating them for vanilloid binding and agonism (Jordt and Julius,

2002). They identified a segment of the rat TRPV1 sequence extending from the second to third transmembrane domains that, when transplanted into the avian ortholog, could reconstitute vanilloid sensitivity. They went on to identify a single amino acid residue, Tyr 511 in the rat sequence, located in the intracellular S2–S3 linker region immediately adjacent to S3, whose mutagenesis resulted in a loss of vanilloid agonism and binding but preservation of TRPV1 activation by protons or heat. Strikingly, in a later study involving the cold-gated ion channel, TRPM8, a similar approach revealed a requirement for a corresponding Tyr residue in the activation of that channel by chemical agonists (Chuang *et al.*, 2004). Thus, the role of this Tyr residue in chemical activation of temperature-gated TRP channels appears to be evolutionarily conserved.

Additional residues also appear to be involved in the interactions between TRPV1 and vanilloid agonists. By analyzing interspecies TRPV1 chimeras and site-directed TRPV1 mutants, several groups convergently identified a residue in the S4 segment (Leu 547 in human and rabbit, Leu 549 in guinea pig, Met 547 in rat) that dictates quantitative differences between mammalian species in the agonist potencies of RTX and phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV) and in the antagonist potency of capsazepine (Chou *et al.*, 2004; Gavva *et al.*, 2004; Phillips *et al.*, 2004). A nearby residue, Thr 550, also appears to contribute to vanilloid binding (Gavva *et al.*, 2004).

Previous structure-activity studies had revealed three important contributors to agonism by classical vanilloid compounds at TRPV1: the so-called "A region" consists of an *o*-methoxy, *p*-hydroxy benzene ring; the "B-region" consists of an amide or reverse amide linkage; and the "C-region" consists of a long hydrophobic domain (fatty acid or phorbol group) (Walpole and Wrigglesworth, 1993). Capsazepine and many other urea-based TRPV1 antagonists exhibit similar, although nonidentical, structural features. Combinations of these findings with the mutagenesis data described earlier in this section and with homology modeling based on the structure of voltage-gated K^+ channels have led to the proposal that the S3 and S4 domains of TRPV1. together with the intervening linker, form a hydrophobic cleft within the channel (Chou et al., 2004). In this cleft, residues in the vicinity of Met 547 and Thr 550 interact with the A region, while Tyr 511 interacts with the hydrophobic C region at the opposite end of these compounds. In addition to the S3-S4 region described previously, there is evidence that other domains of TRPV1 may be essential for vanilloid binding. For example, mutation of Arg 114 or Glu 761, located in the cytosolic amino and carboxyl termini of TRPV1, respectively, results in a loss of vanilloid agonism without an apparent effect on responsiveness to heat (Jung et al., 2002). However, the loss of proton activation in these mutants suggests that these two residues may be playing a more general role in agonist activation, as opposed to being part of the vanilloid binding pocket. Despite these advances, much remains to be learned regarding the structural basis of vanilloid action. Why, for example, does phorbol-containing RTX bind TRPV1 so much more tightly than fatty acid-containing capsaicin? Additional unresolved questions surround the nature of TRPV1-B Region interaction, the mechanistic basis for agonism versus antagonism among vanilloid TRPV1 ligands and the relationship of these findings to the activation of TRPV1 by endocannabinoid compounds.

Mutagenesis studies have also begun to provide clues regarding the activation of TRPV1 by nonvanilloid activators. As indicated in Section III.A., protons both sensitize TRPV1 to its other chemical and thermal stimuli and directly activate TRPV1 at room temperature. In fact, these two effects can be mechanistically dissociated (Jordt *et al.*, 2000). Mutation of Asp 600 results in a loss of the sensitizing effects of protons but preservation of "direct" proton activation. In contrast, mutation of Asp 648 results in a loss of direct proton activation of TRPV1, without eliminating proton-mediated sensitization. In neither case is activation by heat or vanilloid compounds compromised.

To date, no TRPV1 mutation has been described that selectively abrogates heat activation. If the "temperature dependence of voltage gating" hypothesis holds true, one might expect that a charged voltage sensor will be identified and that mutagenesis of this region will inhibit responsiveness to heat, voltage, and possibly other agonists. However, TRPV1 lacks the canonical positively charged residues found in the S4 voltage sensors of classical voltage-gated channels, so that the basis of its voltage sensitivity remains a mystery. One important element of all of these findings is that TRPV1 activation by vanilloid compounds, heat, and protons can be dissociated by mutagenesis of topologically dispersed amino acid residues. This fact rules out a model in which a single agonist sensor domain accounts for TRPV1 by all stimulus modalities, arguing instead for one in which these modalities act through distinct or incompletely overlapping channel subregions. At the same time, however, drugs, such as capsazepine, that act as competitive antagonists of vanilloid binding but still block stimulation by nonvanilloid TRPV1 agonists probably impede conformational changes that are conserved among these disparate activators.

IV. REGULATION OF TRPV1

A critical feature of nociceptive neuronal pathways is their ability to undergo alterations in sensitivity to exogenous noxious input, following acute exposure to noxious stimuli, target tissue inflammation, or direct nerve injury. At the organismal level, these changes are important for two reasons. First, they provide a mechanism for encouraging the protection of injured tissues, to allow those tissues to heal. Second, exaggeration of these mechanisms under pathological conditions accounts for the maladaptive features of chronic inflammatory or neuropathic pain. Ultimately, a rational clinical approach to dealing with maladaptive pain will require a detailed molecular understanding of the sensitization and desensitization mechanisms that occur at the nociceptor terminal.

Activation of TRPV1 produces a classical pattern of acute nociceptor sensitization, followed by a more prolonged desensitization. Fortuitously, many aspects of these processes can be recapitulated in primary neuronal cultures or recombinant expression systems, affording the opportunity to study them in detail.

A. TRPV1 Desensitization

Desensitization of capsaicin-responsive neurons or TRPV1-transfected cells occurs on several timescales, with variable breadth of impact and duration.

1. Acute Homologous TRPV1 Desensitization

Within seconds of capsaicin application under physiological conditions, TRPV1-mediated currents undergo a rapid desensitization and tachyphylaxis that exhibits both Ca²⁺ influx-dependent and -independent components (Docherty et al., 1996). Tominaga, Gordon, and colleagues have provided evidence to suggest that the interaction of TRPV1 with the Ca^{2+} binding protein, calmodulin (CaM), contributes to the Ca²⁺-dependent component of this effect (Numazaki et al., 2003; Rosenbaum et al., 2004). At the whole-cell level, CaM inhibitors abrogate Ca²⁺-dependent TRPV1 desensitization. At the single-channel level, addition of recombinant wildtype CaM but not a Ca²⁺ binding-deficient CaM mutant to the intracellular side of excised membrane patches diminishes TRPV1 open probability in the presence of capsaicin. Two CaM-binding domains have been identified in TRPV1. One is a 35 amino acid region in the cytosolic carboxyl terminus (Numazaki et al., 2003). When expressed alone, this region is sufficient to bind CaM, whereas deletion of this region from TRPV1 results in a channel that fails to exhibit Ca²⁺-dependent desensitization. The other putative CaM-binding domain is a 30 amino acid region overlapping the second ankyrin repeat in the cytosolic TRPV1 amino terminus (Rosenbaum et al., 2004). This domain, when expressed recombinantly, can also bind CaM,

although the consequences of its deletion on desensitization could not be studied, owing to a complete loss of TRPV1 function. Both domains exhibit some binding to CaM in the absence of Ca²⁺. This situation is analogous to that observed in voltage-gated Ca²⁺ channels and suggests that some CaM may be resident on TRPV1 at all times, ideally situated to respond to the rapid influx of Ca²⁺ ions. Intracellular application of the rapid Ca²⁺ chelator BAPTA suppresses Ca²⁺-dependent TRPV1 desensitization, whereas the slower chelator EGTA does not (Docherty *et al.*, 1996). These observations are consistent with the CaM model in which the site of action for Ca²⁺-dependent desensitization is relatively close to the plasma membrane.

It remains unclear how CaM binding to the cytosolic domains of TRPV1 affects channel gating. However, mutation of Tyr 671 to Lys in the sixth transmembrane domain has been shown to eliminate Ca²⁺-dependent desensitization (Mohapatra et al., 2003). Since this residue is unlikely to be exposed to the cytosol, its mutation might interfere with the allosteric transduction of the CaM conformational change to channel domains involved in gating. It will also be interesting to determine whether the bidirectional CaM-dependent regulation (separable desensitization and sensitization processes mediated by the two distinct lobes of CaM) observed in voltage-gated calcium channels by Ca²⁺ will also be seen with TRPV1 (Zuhlke et al., 1999; DeMaria et al., 2001). As discussed in Section IV.B.2., CaM-dependent protein kinase activity may contribute to the reversal of TRPV1 desensitization. Finally, it should be noted that Ca^{2+} -independent forms of TRPV1 desensitization are also observed. These include the desensitization produced by continuous or repeated exposure to heat, which is observed even in the absence of Ca^{2+} (Tominaga *et al.*, 1998). This process also results in cross-desensitization to capsaicin but, for reasons that are not understood, can be partially alleviated by the coadministration of capsaicin and heat.

2. Heterologous Nociceptor Desensitization and Nociceptor Terminal Degeneration

The events outlined previously are observed within a relatively short time frame and are restricted to the activity of the TRPV1 protein, itself. However, one of the hallmarks of capsaicin-mediated desensitization *in vivo* is the inhibition of nociceptor responsiveness to stimuli, such as mechanical force, that are transduced by TRPV1-independent mechanisms. At least two mechanisms contribute to this process. On an intermediate timescale (minutes) TRPV1 activation results in the heterologous desensitization of voltage-gated sodium and calcium channels and consequent block of action potential initiation and propagation at the nociceptor terminal (Bleakman *et al.*, 1990; Liu *et al.*, 2001). The mechanistic basis of this process is not known. With protracted exposure to capsaicin, an even more far-reaching effect is observed. The robust influx of sodium and calcium ions evoked by capsacin results in cytotoxicity at the nociceptor terminal (Jancso, 1992; Simone *et al.*, 1998). Initially, this change can be detected as an increase in mitochondrial Ca^{2+} content, with consequent mitochondrial swelling. Sodium-dependent osmotic cell swelling may also contribute to this effect. Within hours, the nerve terminal undergoes degeneration, completely eliminating its ability to transduce and transmit nociceptive stimuli.

Many features of this process can be recapitulated in nonneuronal cells expressing recombinant TRPV1 (Caterina et al., 1997). In the skin of adult humans or laboratory animals, a single exposure to capsaicin is sufficient to produce visible degeneration and disappearance of epidermal C-fibers at the site of application, together with a local loss of thermal and mechanical nociception (Simone et al., 1998). Over a period of several weeks, there is a partial recovery of both epidermal fibers and nociceptive sensation. Under these circumstances, despite changes in the nociceptor terminals, the TRPV1 positive cell bodies in the dorsal root ganglia remain viable. The transient degeneration of nociceptor terminals in the skin provides the rationale for the use of high-dose topical capsaicin in the treatment of burning sensory neuropathies that are refractory to other treatments (Robbins et al., 1998). However, a local anesthetic must be coadministered with capsaicin under these circumstances, in order to diminish the intense initial burning pain. Despite this side effect, topical capsaicin treatment can produce significant relief in some patients for a period of weeks. An analogous strategy is used in patients with urinary bladder hyperreactivity resulting from spinal cord injury (Chancellor and de Groat, 1999). The urinary bladder is rich in capsaicin-sensitive afferents, and these fibers are thought to contribute to the urinary urgency, urinary frequency, and bladder discomfort experienced by such patients. In a significant fraction of these individuals, intravesical administration of capsaicin or RTX has been shown to provide prolonged but reversible relief of bladder symptoms.

In contrast to the reversible effects of topical capsaicin treatment described earlier, if high concentrations of capsaicin are administered systemically to rats during the neonatal period, small-diameter nociceptive neurons are killed, resulting in a lifelong reduction in noxious thermosensation, neurogenic inflammation, and in some cases, mechanical nociception (Jancso *et al.*, 1967; Scadding, 1980). As an extension of this approach, RTX administered directly to peripheral sensory ganglia has been used to permanently ablate small-diameter nociceptive neurons in adult rats and arthritic dogs (Karai *et al.*, 2004a).

B. TRPV1 Sensitization

Another aspect of TRPV1 regulation that has received considerable attention concerns the mechanisms by which the inflammatory mediators that bathe nociceptor terminals in injured tissues sensitize TRPV1 to its physical and chemical agonists. The "inflammatory soup" produced during tissue injury contains a variety of factors that are capable of sensitizing nociceptor terminals to noxious thermal, chemical, and mechanical stimuli (Handwerker and Reeh, 1991). At least some of this sensitization appears to involve TRPV1. Whereas some inflammatory factors (e.g., protons) act directly on the capsaicin receptor, others influence this channel indirectly. The latter group includes: neurotrophins (e.g., NGF, NT-3, BDNF, GDNF), which act via receptors with intrinsic tyrosine kinase activity (Shu and Mendell, 1999; Chuang et al., 2001; Bonnington and McNaughton, 2003; Galoyan et al., 2003; Prescott and Julius, 2003); adenosine triphosphate, bradykinin, proteases, prostaglandin E2, and glutamate, which act via G-protein-coupled receptors (Lopshire and Nicol, 1998; Chuang et al., 2001; Vellani et al., 2001; Bhave et al., 2002; Moriyama et al., 2003; Amadesi et al., 2004; Dai et al., 2004); and cytokines (e.g., interleukin 1β , tumor necrosis factor a, interleukin-6), which act via cell-surface receptors coupled to the JAK/STAT signaling pathway (Opree and Kress, 2000). TRPV1 sensitization can occur in a number of ways.

1. Some Inflammatory Mediators Trigger the Production of TRPV1 Agonists

One mechanism by which heterologous stimuli sensitize TRPV1 is by stimulating the production and release of TRPV1 agonists. An example of this effect is the G-protein-mediated synthesis and release of 12-HPETE that is triggered by the binding of the proalgesic peptide, bradykinin, to the B2 receptor subtype (Shin *et al.*, 2002; Ferreira *et al.*, 2004). In this case, activation of diacylglycerol lipase results in the cleavage of arachidonic acid from phospholipids and the conversion of this fatty acid to 12-HPETE. Theoretically, this membrane-permeant lipoxygenase product can then access TRPV1 in the same or adjacent terminals.

2. TRPV1 can also be Sensitized by Phosphorylation

A second mechanism by which the TRPV1 protein can be sensitized heterologously is through phosphorylation of its cytosolic domains. Protein kinase C (PKC) (Premkumar and Ahern, 2000; Vellani *et al.*, 2001; Bhave *et al.*, 2003; Moriyama *et al.*, 2003; Dai *et al.*, 2004) and cyclic AMP-dependent protein kinase (PKA) (Lopshire and Nicol, 1998; Bhave *et al.*, 2002) have both been shown to directly phosphorylate TRPV1 at a number

of serine and threonine residues (Fig. 3). These kinases are activated by G-protein-coupled signaling pathways downstream of the receptors for such inflammatory mediators as ATP (P2Y2 receptors), bradykinin (BK2 receptors), prostaglandin E2, glutamate, and even proteases (PAR2 receptors). Phosphorylation of TRPV1 by PKA or PKC has been shown to enhance channel sensitivity to agonists, such as capsaicin and heat, or to reverse channel desensitization. Another cellular kinase, Ca²⁺/Calmodulin-dependent protein kinase type II (CaMKII) has also been shown to reverse TRPV1 desensitization, and essential serine and threonine residues for this process have been identified (Jung et al., 2004). However, direct phosphorylation of TRPV1 by this enzyme has yet to be demonstrated. Likewise, as described in Section IV.B.3., certain mitogen activated protein kinases can influence TRPV1 localization or expression. However, here again, evidence for direct phosphorylation of TRPV1 by these proteins has not yet emerged. A more extensive description of regulation of TRPV1 by phosphorylation is provided in Chapter 6 of this volume.

3. Nerve Growth Factor may Sensitize TRPV1 by Multiple Mechanisms

Among the best-studied sensitizers of TRPV1 is the neurotrophin, nerve growth factor (NGF). Studies have led to the recognition that NGFdependent enhancement of TRPV1 function is multifaceted, occurring over several different timescales and by means of several different mechanisms. The most fundamental way in which NGF enhances capsaicin and heat sensitivity is by promoting TRPV1 mRNA expression during development. This effect can be recapitulated in cultured, isolated nociceptive neurons from neonatal rodents. In the adult rat, however, Woolf and colleagues have shown that NGF-induced increases in TRPV1 protein at peripheral nociceptor terminals are largely the result of posttranscriptional mechanisms, most likely including enhanced TRPV1 translation and selective translocation to peripheral nociceptor terminals (Ji et al., 2002) (Fig. 5). This effect appears to occur via the ras-dependent activation of the p38 MAP kinase pathway. It has also been recognized that NGF can sensitize existing TRPV1 molecules on a timescale of several minutes (Shu and Mendell, 1999).

At least two mechanisms have been proposed for this acute sensitization. One is through the degradation of PIP₂ (Chuang *et al.*, 2001; Prescott and Julius, 2003). As described earlier in Section III.B, there is evidence that, under basal conditions, the binding of this phospholipid to TRPV1 produces tonic channel inhibition. The precise requirements for PIP₂ binding are not known, but it has been shown to depend upon a stretch of cationic amino acids located in the distal portion of the TRPV1 carboxyl terminus.

Binding of NGF to its receptor, TrkA, results in the activation of phospholipase C γ , which cleaves PIP₂. As an apparent consequence of this event, the TRPV1 threshold for thermal activation is reduced from \sim 42°C to well below body temperature, its temperature response profile becomes more shallow, and its sensitivity to capsaicin increases. These effects on TRPV1 can be mimicked by chelation of PIP₂ with selective monoclonal antibodies or removal of the putative PIP₂ binding domain and does not depend on activation of PKC. The requirement reported for PLC in acute NGF sensitization of TRPV1 (Galoyan et al., 2003) provides further support for this mechanism. Perhaps the most compelling demonstration of the importance of this interaction is that if the putative PIP₂ binding site of TRPV1 is replaced with the high-affinity PIP_2 binding domain from an inwardly rectifying K^+ channel, IRK1, the thermal threshold for TRPV1 activation is shifted to higher temperatures (Prescott and Julius, 2003). Thus, PIP₂ binding and its reversal by PIP₂ cleavage, like phosphorylation/dephosphorylation, appears to represent a mechanism for fine-tuning of TRPV1 agonist responsiveness.

A second acute mechanism for NGF-mediated sensitization of TRPV1 is the TrkA-dependent activation of phosphatidyl inositol 3-kinase (PI3 Kinase) to generate phosphatidyl 3,4,5 trisphosphate (PIP₃) (Bonnington and McNaughton, 2003; Zhuang *et al.*, 2004). Pharmacological inhibition of this signaling enzyme blocks acute TRPV1 sensitization by neurotrophins through a mechanism that appears to involve PKC and CaM kinase II but not protein kinase A or phospholipase C. Curiously, information also appears to flow in the opposite direction, as capsaicin activation of TRPV1 in cultured sensory neurons leads to PI3 Kinase-dependent activation of ERK and protein kinase B (Zhuang *et al.*, 2004). These findings, together with the observation that TRPV1 and TrkA appear to exisit in a macromolecular complex, suggest the existence of highly coordinated interactions among these signaling molecules.

4. Direct TRPV1 Sensitization by Heat

While not a by-product of inflammation, intense heat, itself, can sensitize TRPV1 to further thermal challenge. If sensory neurons are heated to temperatures >50°C, the temperature-response curve of heat-gated currents is shifted to much lower temperatures with a ~threefold reduction in Q10 (Lyfenko *et al.*, 2002). This effect appears to be relatively long lasting and has been suggested to reflect partial denaturation of the TRPV1 protein structure. A similar phenomenon is exhibited by recombinant TRPV1 expressed in Xenopus oocytes (Caterina *et al.*, 1999). *In vivo*, this phenomenon might contribute to burn injury-induced thermal hyperalgesia.

C. Convergence of Sensitizing and Desensitizing Influences on TRPV1

As indicated in Fig. 3, there is considerable proximity and overlap among the sites that regulate TRPV1 activity. For example, the putative PIP₂ binding domain, one of the CaM binding domains, and the PKC phosphorvlation site Ser 800 all occur within the same segment of the TRPV1 C-terminus. Also, as described earlier in Section III.B.1., TRPV1 sensitization by phosphorylation appears, in some cases, to reverse Ca²⁺-dependent desensitization. Accordingly, inhibition of the serine/threonine phosphatase. calcineurin, prevents Ca2+-dependent TRPV1 desensitization (Docherty et al., 1996; Jung et al., 2004). Moreover, truncation of the last 72 amino acids from rat TRPV1 results in a channel with enhanced sensitivity to heat (lower apparent threshold temperature for activation) but a more shallow temperature-response profile (Vlachova et al., 2003). This finding is consistent with a role for the TRPV1 C-terminus in suppression of channel activity and therefore provides a potential rationale for the convergence among the sensitizing and desensitizing inputs in a small number of intracellular regulatory regions.

V. CONTRIBUTIONS OF TRPV1 TO ACUTE NOCICEPTION AND HYPERALGESIA

The prominent expression of TRPV1 in unmyelinated small-diameter neurons and its activation by noxious chemical and physical stimuli make this channel a compelling candidate transducer of painful stimuli in vivo. Among the array of stimuli that can excite nociceptors, capsaicin, protons, and noxious heat have attracted considerable attention because of their robust actions on these cells (Kress and Zeilhofer, 1999). Currents elicited by these three noxious stimuli in sensory neurons share many features with the TRPV1-mediated currents observed in heterologous expression systems, with respect to kinetics, ionic composition, and pharmacological properties. In addition, the analysis of sensory neurons isolated from mice in which the TRPV1 gene has been disrupted has demonstrated a requirement of this channel protein for the normal transduction of each of these stimuli. In the cases of vanilloid and heat responsiveness, a requirement for TRPV1 has also been observed at the level of intact skin-nerve explants, in *in vivo* spinal cord recordings and even in behavioral assays in awake mice. As outlined in the following sections, however, whereas TRPV1 is absolutely essential for nociceptive responses to capsaicin, there is evidence for a substantial contribution from TRPV1-independent processes to acute heat nociception.

A. Endogenous TRPV1 and the Detection of Vanilloid Compounds by Nociceptors

Capsaicin sensitivity, which was the basis of the strategy used to clone TRPV1, has been viewed by some as the best functional marker for the C-fiber nociceptor (Holzer, 1991). In vitro, capsaicin excites approximately half of primary sensory neurons cultured from dorsal root or trigeminal ganglia by evoking a nonselective cationic current with high permeability to Ca²⁺ (Williams and Zieglgansberger, 1982; Baccaglini and Hogan, 1983; Heyman and Rang, 1985; Marsh et al., 1987; Winter, 1987; Bleakman et al., 1990; Bevan and Docherty, 1993; Oh et al., 1996). As described earlier in Section I.B., recombinant TRPV1 faithfully recapitulates these currents at the whole-cell and single-channel levels. In addition, pharmacological agents, such as capsazepine, ruthenium red, and SB366791, that block endogenous capsaicin-evoked currents invariably block vanilloid-evoked currents mediated by recombinant TRPV1 and do so with similar order of agonist potency (Rami and Gunthorpe, 2004). Most strikingly, in sensory neurons derived from TRPV1 null mutant mice, capsaicin- and RTX-evoked currents are completely absent from either IB4⁺ or IB4⁻ neurons (Caterina et al., 2000; Davis et al., 2000) (Fig. 4). Consistent with these findings, there is an absence of capsaicin sensitivity in skin-nerve explants derived from TRPV1 null mice (Caterina et al., 2000). In vivo, multiple TRPV1 antagonists have been shown to inhibit acute behavioral nocifensive responsiveness to capsaicin and/or capsacin-evoked mechanical hyperalgesia (Garcia-Martinez et al., 2002; Pomonis et al., 2003; Walker et al., 2003). Finally, TRPV1 gene disruption leads to the disappearance of nocifensive behaviors in mice injected with intraplantar capsaicin or RTX, a virtual elimination of capsaicin-evoked neurogenic inflammation and a lack of avoidance of capsaicin-containing drinking water (Caterina et al., 2000; Davis et al., 2000) (Fig. 4). Together, these findings make it clear that TRPV1 is both necessary and sufficient for responses to vanilloid compounds, although they cannot definitively exclude the participation of other proteins in conjunction with TRPV1.

B. Endogenous TRPV1 and the Detection of Protons by Nociceptors

Protons activate at least two distinct current types that differ in their kinetics, pH dependence of activation and inactivation, and ionic selectivities. The first of these is transient, with fast activation and inactivation and high selectivity for Na⁺ ions (Konnerth *et al.*, 1987; Krishtal *et al.*, 1988). This current type appears to be mediated by amiloride-sensitive



FIGURE 4 In vitro and in vivo consequences of TRPV1 gene disruption. (A) Requirement of TRPV1 for responsiveness to vanilloid compounds. Top, Current responses of cultured DRG neurons derived from wild-type (+/+) and TRPV1 null (-/-) mice to capsaicin $(1 \ \mu M)$ and RTX (300 nM)

channels of the Acid-sensing ion channel (ASIC) family, which are widely expressed in the nervous system and whose gene disruption leads to a reduction in certain forms of acid-evoked hyperalgesia (Waldmann et al., 1999; Price et al., 2001; Xie et al., 2002; Sluka et al., 2003). The second proton-evoked current type is more sustained and is observed solely in nociceptive neurons (Kress and Zeilhofer, 1999). These sustained currents, which are often coincident with capsaicin sensitivity and resemble protonevoked TRPV1 currents in many respects (Tominaga et al., 1998), might contribute to the persistent pain sensation associated with local tissue acidosis. While certain ASIC channels, alone or in combination, exhibit a sustained, Ca²⁺-containing current component, they probably cannot account for the bulk of the sustained proton-evoked currents observed at pH 5. Indeed, the sustained proton-evoked currents observed under such moderately acidic conditions were found to be dramatically reduced in prevalence among sensory neurons from mice lacking TRPV1 (Caterina et al., 2000; Davis et al., 2000). A similar dramatic reduction was observed in the prevalence of acid-sensitive C-fibers in skin-nerve explants derived from these mice (Caterina et al., 2000). Thus, TRPV1 appears to be critical for at least a component of nociceptor acid responsiveness in vitro. In the intact animal, however, deficits in acute acid-evoked nociception have yet to be reported in mice lacking TRPV1.

C. Endogenous TRPV1 and Nociceptor Responses to Heat

On exposure to noxious temperatures, a specific subpopulation of cultured neurons from rat or mouse dorsal root or trigeminal ganglia responds with robust inward currents that carry Ca^{2+} ions and exhibit an outwardly rectifying current–voltage relationship (Cesare and McNaughton, 1996; Kirschstein *et al.*, 1997; Reichling and Levine, 1997; Nagy and Rang, 1999a). However, these responses exhibit a certain degree of heterogeneity.

under voltage-clamp at -80 mV. Proportion of cells exhibiting the indicated responses is indicated at right. Bottom, Paw licking behavior evoked by the intraplantar administration of capsaicin (1 µg) RXT (0.05 n moles) or vehicle in wild-type versus TRPV1 null mice. (B) Requirement of TRPV1 for normal responsiveness to heat. Top, Current responses of cultured DRG neurons derived from wild-type (+/+) and TRPV1 null (-/-) mice to the indicated heat ramps. Proportion of cells exhibiting indicated response type is indicated. Bottom Left, Comparison of heat-evoked action potential responses among C-fibers in isolated skinnerve explants. In this experiment, 13/24 wild-type C-fibers and 4/24 TRPV1 null C fibers exhibited measurable heat-evoked increases in firing rate. Bottom Right, comparison of tail withdrawal latencies of wild-type and TRPV1 null mice from a water bath set at the indicated temperatures. Data were reproduced from Caterina *et al. Science* **288**, 217–388, copyright 2000, with permission from The American Association for the Advancement of Science.

Approximately 40% of sensory neurons exhibit a thermal threshold of \sim 42–45°C. These neurons also tend to be capsaicin sensitive. In contrast, $\sim 10\%$ of neurons exhibit a much higher activation threshold of $\sim 52^{\circ}$ C and are largely capsaicin-insensitive (Nagy and Rang, 1999a) (Fig. 4). These in vitro findings present a striking parallel to the in vivo observation, made in primates, that C-fibers and type I A δ fibers tend to exhibit thermal activation thresholds of 42–45°C, whereas type II A δ fibers are activated only at >52°C (Dubner et al., 1977; Campbell and Meyer, 1986). Consistent with its thermal response properties, TRPV1 appears to be required for the 42°C threshold in vitro responses of cultured nociceptive neurons. Not only are these responses inhibited by capsazepine (Kirschstein et al., 1999; Nagy and Rang, 1999b) but also are totally absent among neurons derived from mice lacking TRPV1 (Caterina et al., 2000; Davis et al., 2000) (Fig. 4). The higher-threshold heat-evoked currents, in contrast, are unaffected in these animals, suggesting the presence of additional thermotransduction mechanisms.

There is considerable additional evidence that TRPV1 plays a significant but partial role in acute thermal nociception. Skin-nerve explants derived from TRPV1 null mice, exhibit a partial reduction in both the proportion of heat-sensitive C-fibers and the mean heat-evoked firing frequencies among those heat-sensitive fibers that are observed (Caterina et al., 2000) (Fig. 4). In contrast, in a skin-DRG-spinal cord explant preparation, no differences in heat sensitivity were observed between C-fibers from wild-type versus TRPV1 null mice (Woodbury et al., 2004). In vivo, electrophysiological recording from wide dynamic range neurons in the spinal cord dorsal horn as well as immunohistochemical analysis of heat-evoked spinal cord c-fos induction have revealed profound deficits in TRPV1 null mouse responsiveness to heating of the plantar skin of the hind paw. At the behavioral level, TRPV1 null mice exhibit significant increases in withdrawal latencies from thermal stimuli in the tail immersion, hot plate, and radiant paw heating assays, which are most pronounced at relatively high stimulus temperatures (>50°C) (Caterina et al., 2000) (Fig. 4). Another group failed to observe statistically significant alterations in hot plate latency in a different line of TRPV1 null mice (Davis et al., 2000). However, the range of stimulus temperatures explored in that study was relatively restricted. Moreover, additional support for a role of TRPV1 in acute thermal nociception has come from the observation of reduced behavioral responsiveness to heat in wild-type mice treated with N-alkyl glycine-derived TRPV1-selective antagonists (Garcia-Martinez et al., 2002).

Taken together, these findings strongly suggest that, whereas TRPV1 is certainly a contributor to acute heat-evoked nociception, other heat transducers are also likely to play a role and may compensate for TRPV1 absence

or blockade in certain behavioral or electrophysiological assays. What might these other transducers be? The best candidates, at present, are a group of ion channel subunits homologous to TRPV1. TRPV2, TRPV3, and TRPV4 are heat-gated channels that can be activated at temperatures of >52°C, >34–39°C, and >27–34°C, respectively (Benham *et al.*, 2003). TRPV2 is expressed most highly in a subset of medium- to large-diameter myelinated sensory neurons, making it a strong candidate mediator of the "highthreshold" heat-evoked currents observed in capsaicin-insensitive neurons in culture and in type II A δ neurons *in vivo*. TRPV3 and TRPV4 are probably expressed at some level in sensory neurons but are expressed at an even higher level in skin keratinocytes. These findings have led to the suggestion that keratinocytes, by signaling to nearby sensory afferents, may mediate certain aspects of innocuous and/or noxious thermosensation. Gene knockout studies, currently in progress, should allow these possibilities to be tested directly.

D. TRPV1 and Thermal Hyperalgesia Following Inflammation

There is substantial evidence that, besides contributing to acute thermal nociception, TRPV1 plays an essential role in the thermal hyperalgesia that follows peripheral inflammation. Evidence in support of this claim comes from multiple sources. First, as described previously, many mediators produced in response to tissue inflammation are capable of sensitizing TRPV1 to its thermal and chemical activators. Second, TRPV1 protein expression and localization are dramatically altered following inflammation. Local inflammation caused by injection of Complete Freud Adjuvant (CFA) onto the hinpaw of rats, for example, has been shown to increase both the absolute TRPV1 expression level and proportion of TRPV1 immunopositive cells among unmyelinated DRG neurons (Amaya et al., 2000; Carlton and Coggeshall, 2001). Other investigators have reported that inflammation also causes a substantial increase in the proportion of TRPV1-positive myelinated fibers (Luo et al., 2004). At least one mechanism for these increases appear to be posttranscriptional, since CFA injection into the rat hindpaw induces the accumulation of the TPRV1 protein level in cutaneous nerve endings and DRG cell bodies but not in the central projections to the spinal cord dorsal horn (Ji et al., 2002) (Fig. 5). Third and most strikingly disruption of the TRPV1 gene in mice leads to a virtual ablation of behavioral thermal hyperalgesia resulting from a wide range of inflammatory stimuli, including CFA, carageenan, and mustard oil, as well as defined inflammatory mediators such as NGF, bradykinin, and protease receptor ligands (Caterina et al., 2000; Davis et al., 2000; Chuang et al., 2001; Amadesi et al.,



FIGURE 5 TRPV1 is critical for inflammatory thermal hyperalgesia. (A) Increased TRPV1 immunoreactivity observed in the rat sciatic nerve following intraplantar administration of CFA. (B) Immunoblot comparison of TRPV1 protein levels in the plantar skin, sciatic nerve, and spinal cord following CFA treatement. Inhibition of TRPV1 upregulation by a p38 MAP kinase inhibitor (SB203580, 1 μ g, twice per day \times 2 days, intrathecal) is also indicated. (C) Failure of CFA to produce a shortened latency of paw withdrawal from a radiant heat source in mice lacking TRPV1. (D) Mice lacking TRPV1 also exhibit virtually no change in hot plate latency following the application of mustard oil to the hind paw. Panels A and B reprinted from Ji *et al.*, *Neuron* **36**, 57–68, copyright 2002, with permission from Elsevier. Panels C and D reproduced from Caterina *et al. Science* **288**, 217–388, copyright 2000, with permission from The American Association for the Advancement of Science.

2004; Dai *et al.*, 2004) (Fig. 5). Consistent with these findings, several TRPV1 antagonists (*N*-alkyl glycine derivatives, BCTC, and capsazepine) dose-dependently inhibit the thermal hyperalgesia caused by mustard oil or CFA (Garcia-Martinez *et al.*, 2002; Pomonis *et al.*, 2003; Walker *et al.*, 2003). In the latter case, whereas capsazepine was effective at blocking thermal hyperalgesia in the guinea pig, it was not effective in the rat, most likely due to the species variability in capsazepine sensitivity outlined in Section III.B.

E. TRPV1 and Thermal Hyperalgesia Following Nerve Injury

In contrast to the situation for inflammatory thermal hyperalgesia, there is no evidence yet that TRPV1 contributes to the thermal hyperalgesia associated with neuropathic pain states. This conclusion stems largely from the observation that mice lacking TRPV1 exhibit an apparently normal degree of thermal hyperalgesia following partial sciatic nerve ligation. However, TRPV1 expression levels increase in uninjured afferents following L5 spinal nerve ligation (Fukuoka *et al.*, 2002). Thus, this issue needs to be further explored pharmacologically or using alternative neuropathic models.

F. Role for TRPV1 in Mechanical Nociception and Mechanical Hyperalgesia

One of the more surprising findings to arise from the in vivo study of TRPV1 antagonists is that in at least two cases (BCTC in rats and capsazepine in guinea pigs), administration of these drugs has led to a reduction in mechanical hyperalgesia resulting from partial sciatic nerve ligation (Pomonis et al., 2003; Walker et al., 2003). These findings are in apparent contrast to the normal baseline mechanical nociception and normal mechanical allodynia reported in TRPV1 null mice following the same neuropathic insult (Caterina et al., 2000). They are also somewhat counterintuitive, given that heterologous expression of TRPV1 does not apparently confer mechanosensitivity to cells. The possibility cannot be excluded that TRPV1 is one component of the mechanosensory apparatus of nociceptors and that other molecules compensate for its absence in the knockout mice. Indeed, mechanical responsiveness in the urinary bladder appears to be diminished in mice lacking TRPV1 (Birder et al., 2002). However, it is also possible that the apparent efficacy of BCTC and capsazepine result not from the antagonism of TRPV1 but rather from their already-documented ability to act on other channels. The *in vivo* application of highly selective TRPV1 antagonists will be required in order to resolve this issue definitively.

VI. CONCLUDING REMARKS

In some ways, TRPV1 can be viewed as a proteinaceous microcosm of the nociceptor terminal. It responds in a graded manner to diverse physical and chemical stimuli, distinguishes painful from nonpainful inputs, and changes its responsiveness as a function of its history and chemical milieu. However, the findings outlined in this chapter make it clear that this channel is one component of a much larger repertoire of transduction mechanisms available to nociceptors. In addition, while we now know a fair bit about the pharmacological and physiological properties of TRPV1, much remains to be learned. How exactly do chemical ligands or heat enhance channel opening? With what other proteins does TRPV1 collaborate to perform its cellular functions? Most importantly, which clinical conditions, if any, will respond best to TRPV1 antagonists? Given the brisk pace at which this field is growing, the answers to these questions should not be long in coming.

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CHAPTER 5

Nociceptive Signals to TRPV1 and its Clinical Potential

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References

I. INTRODUCTION

Nociceptive neurons are a peripherally located subset of sensory neurons that transmit pain signals to the spinal cord. Pain signal conducting sensory neurons are classified as unmyelinated or as small-myelinated nerve fibers, as compared with the large, rapidly conducting fibers that transmit tactile information. Nociceptive (painful) signals originate in nociceptive sensory neuron terminals, where many ion channels and receptors are expressed to transduce various stimuli to neural signals. These receptors or channels are activated by specific stimuli and serve as molecular sensors. Advances in basic biomedical technology have found that many ion channels are specifically activated by different types of stimuli. Of the channels discovered to date, TRPV1 has been studied most extensively because of its functional significance in the pain sensory system. In the present chapter, different types of endogenous activators or endovanilloids are introduced, and structural comparisons between endovanilloids and capsaicin are discussed. Mutagenic studies on TRPV1 have identified three ligand-binding sites. One of these sites is located in transmembrane domain 3 and the other two are represented by Arg 114 and Glu 761 in N- and C-termini, respectively. The critical region in transmembrane domain 3 is conserved in the capsaicin-sensitive orthologs of some mammalian TRPV1s, whereas the latter two sites are highly conserved throughout species whether or not the TRPV1 orthologs are sensitive to capsaicin.

Because TRPV1 is implicated in the mediation of inflammatory pain, we discuss the signaling pathways that link TRPV1 and inflammatory mediators such as bradykinin (BK) and histamine. Because of its implication as a molecular target for a new class of analgesics, TRPV1 antagonists have drawn the attentions of pharmaceutical companies as development bases for potential analgesics. In the present chapter, we introduce some newly developed TRPV1 antagonists.

A. Effect of Capsaicin on Sensory Neurons

Hot chili peppers are a popular food additive. They were first grown in South America, and were transported to the Western World during the 15th century (Szolcsanyi, 1993). The major ingredient of peppers when first extracted was called "capsicol," which was then described to have a "neuro-selective" action because of its specificity for sensory nerves (Szolcsanyi, 1993). The canonical action of capsaicin in terms of its excitation of sensory nerves was well described by a group of Hungarian scientists,

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Jancso and Szolcsanyi (see also Chapter 4). Capsaicin, a vanilloid analog, as it possesses the vanillin moiety (Fig. 1), causes severe pain and pain-related reflexes in man and animals (Simone *et al.*, 1987, 1989). The pain elicited by capsaicin is due to its excitatory action on sensory neurons. Capsaicin excites a subset of sensory nerve fibers, especially, unmyelinated (C-) or small myelinated ($A\delta$ -) fibers that are considered to mediate the sensation of pain (Szolcsanyi, 1993). Thus, capsaicin is also called an "excitotoxin" or a "neurotoxin" (Winter *et al.*, 1990; Oh *et al.*, 1996; Holzer, 1998).



FIGURE 1 Structures of capsaicin, vanillin, 12-HPETE, and endovanilloids.

The excitation of sensory neurons by capsaicin suggests the presence of cation channels that are activated by capsaicin. In early biochemical studies it was found that capsaicin caused influxes of Rb^+ or Ca^{2+} in cultured dorsal root ganglia (Wood *et al.*, 1988). The actual currents activated by capsaicin were first described by Bevan and Szolcsanyi (Bevan and Szolcsanyi, 1990), who demonstrated that capsaicin opens a nonselective cation channel because capsaicin induces inward currents and a concomitant increase in membrane conductance that reverses at a membrane potential of 0 mV (Bevan and Szolcsanyi, 1990). Subsequently, Bevan and Docherty reported that capsaicin induced inward currents are dose-dependent (Bevan and Docherty, 1993). Furthermore, in this same study it was found that the acidification of extracellular fluid also caused inward currents in the sensory neurons. Later, Liu and Simon (1994) confirmed that inward currents are induced by capsaicin in trigeminal neurons.

B. Biophysical Properties of Capsaicin-Activated Channels

A comprehensive description of characteristics of capsaicin-activated currents was obtained from single-channel current recordings produced using patch-clamp techniques. Initially, Bevan and his colleagues produced traces of single-channel currents in sensory neurons activated by capsaicin (Bevan and Docherty, 1993). Later, a complete description of single-channel currents activated by capsaicin was presented by Oh and colleagues (Oh et al., 1996). According to this study, capsaicin-activated channels are permeable to various monovalent cations, namely, Na⁺, K⁺, Cs⁺, and Ca²⁺. Moreover, single-channel conductances were found to be 45 pS and 80 pS at -60 and +60 mV, respectively, when Na⁺ is the major charge carrier (Oh et al., 1996). Thus, single-channel currents activated by capsaicin are known to be outwardly rectifying, which was later found to be a universal feature of TRPV channels. One important finding of the study was that the channel involved was a ligand-gated ion channel as channel currents were observed in isolated membrane patches. Capsaicin-activated channel is also weakly dependent on membrane potential. Channel open probability (P_{0}) is much greater at depolarized than at hyperpolarized potentials. Capsaicin channel is activated in patches when depolarized without capsaicin application (unpublished data) with a half-maximal voltage of -8.8 mV. Furthermore, for every 28.8 mV increase in membrane potential, an *e*-fold increase in P_0 is observed (Oh *et al.*, 1996). Voltage dependence was later confirmed in TRPV1, a cloned capsaicin receptor, and is now considered a universal property of the TRPV subfamily (Nilius et al., 2004).

C. TRPV1: A Cloned Capsaicin-Activated Channel

After a full description of capsaicin-activated channels in sensory neurons was made available (Oh et al., 1996), David Julius and his colleagues at UCSF cloned a gene in a rat model, using functional expression cloning technique, which encoded a capsaicin-activated channel (Caterina et al., 1997). The cloned gene was initially named vanilloid receptor 1 (VR1), because it was activated by capsaicin or resiniferatoxin (RTX), the vanilloid analogs (Caterina et al., 1997). VR1 was later termed TRPV1 according to the new taxonomy of TRP channels (Montell et al., 2002; Clapham, 2003). Rat TRPV1 encodes an 838 amino acid protein of molecular weight \sim 95 kDa. The predicted topology of TRPV1 shows that it has six transmembrane domains and two intracellular cytosolic tails in N- and Ctermini with three ankyrin repeats in the N-terminus (Fig. 3) (see also Chapters 4 and 6). To our surprise, the cloned channel was found to have near identical channel properties to those of native capsaicin-activated channel in sensory neurons, that is, in terms of channel conductance, ion selectivity, and current-voltage relationship (Caterina et al., 1997; Shin et al., 2001). The most striking and interesting aspect of TRPV1 is its activation by heat and acid in addition to capsaicin (Tominaga et al., 1998). Thus, TRPV1 is capable of detecting several noxious stimuli, such as heat and acid, and is referred to as a "polymodal" molecular sensor that transduces these adverse stimuli to nociceptive neural signals in sensory neurons.

D. Capsaicin Binds TRPV1 from the Cytosolic Side

Another property unique to TRPV1 is its activation via intracellular capsaicin. Unlike many other ligand-gated ion channels, capsaicin acts on TRPV1 from the cytosolic side. However, normally capsaicin is applied to a bath of outside-out patches or to whole cells (extracellular side), though it is assumed that binding occurs at the extracellular side (Oh *et al.*, 1996; Jung, 1999). Thus, capsaicin appears to act on TRPV1 from both sides of the channel. However, this is only made possible by the highly lipophilic nature of capsaicin, which easily traverses the cell membrane, to access binding sites from the intracellular side. Moreover, this lipophilic nature made it difficult to determine initially whether capsaicin binds TRPV1 intracellularly or extracellularly. Luckily, Jung *et al.* (1999) found a water-soluble analog of capsaicin, DA-5018•HCl, which cannot cross the plasma membrane easily. When this hydrophilic analog of capsaicin was applied to the intracellular

side in sensory neurons, it produced single-channel currents in a capsazepine-reversible manner. However, DA-5018HCl fails to activate capsaicin channel when applied to the extracellular side (Jung *et al.*, 1999), thus, demonstrating that capsaicin and its analogs act on the intracellular side of TRPV1. This finding becomes important for determining the location of ligand-recognition sites or identifying intracellular signals upstream of TRPV1 (see in a later section).

II. ENDOGENOUS ACTIVATORS OF TRPV1

A. Anandamide

Many efforts have been made to identify the endogenous activators of TRPV1. The first candidate was anandamide (Fig. 1), which was initially isolated from pig brain as an endogenous ligand of cannabinoid receptor (CB1), also known marijuana receptors (Devane et al., 1992; Howlett, 1995). Anadamide is an arachidonic acid (AA) metabolite that is cleaved from N-arachidonyl phosphatidylethanolamine by a phosphodiesterase (Devane and Axelrod, 1994; Cadas et al., 1997) and is known to relax vascular smooth muscles via capsaicin-sensitive nerve fibers (Zygmunt et al., 1999). Furthermore, the application of anandamide to cloned VR1 elicits capsazepine-reversible currents, thus suggesting that anandamide directly activates VR1. Another endocannabinoid, 2-arachidonylglycerol, produces a weaker current response than anandamide when applied to TRPV1 expressed in HEK cells (Zygmunt et al., 1999). The action of anandamide on TRPV1 has been shown by many studies. For example, anandamide induces apoptosis by interacting with VR1 in various cancer cells in a manner identical to that of capsaicin (Maccarrone et al., 2000; Shin et al., 2003; Contassot, 2004). Thus, it is evident that anandamide activates TRPV1.

However, its action on sensory neurons is somewhat puzzling. Cannabinoid receptors are expressed in sensory neurons and are known to be more or less related to antinociception, because anandamide when administered intradermally inhibits formalin-evoked nociception (Calignano *et al.*, 1998). Moreover, the antinociceptive effect of anandamide is blocked by CB1 receptor antagonist (Calignano *et al.*, 1998), whereas the antinociceptive effects of endocannabinoids appear tonic *in vivo*, because the systemic application of a CB1 antagonist evokes hyperalgesic responses (Calignano *et al.*, 1998). Substantial amounts of anandamide are present in the periphery. Furthermore, both CB1 and TRPV1 receptors are expressed in the same sensory neurons. For example, more than 80% of CB1-expressing cells also show positive VR1-like immunoreactivity and *vice versa* (Ahluwalia *et al.*, 2000). Thus, the physiological role of anandamide in terms of TRPV1 activation in sensory neurons is unclear. However, the actions of anandamide on CB1 and TRPV1 are somewhat clearer in the vascular system. As was initially found in vascular smooth muscles (Zygmunt *et al.*, 1999), anandamide induces hypotension by activating CB1 receptor and TRPV1 to similar extents. Moreover, anandamide may have physiological significance in brain functions that involve the hypothalamus or hippocampus, because it depresses paired pulse field potentials when applied to hippocampal slices via TRPV1 but not CB1 receptor (Al-Hayani *et al.*, 2001). Thus, it appears plausible that anandamide plays a greater role in the vascular system or central nervous system (CNS) than in peripheral nociceptors.

B. N-Arachidonyl-Dopamine

Another AA metabolite, N-archidonyldopamine (NADA) (Fig. 1) was suggested to be an endogenous capsaicin-like substance (Huang et al., 2002). The candidacy of NADA as an endovanilloid is more plausible because it has putative vanilloid-like moiety that is lacked in anandamide (Fig. 1). Di Marzo and his colleagues sought to determine whether NADA actually activates TRPV1 (Huang et al., 2002). In a Ca²⁺ imaging study, NADA was found to induce an influx of Ca^{2+} in human embryonic kidney (HEK) cells transfected with TRPV1. The concentration of NADA required to induce this Ca^{2+} -influx was about 48 nM, which is almost equal to that of capsaicin determined by Ca^{2+} imaging, but which is about 10 times more potent than anandamide (Huang et al., 2002; De Petrocellis, 2004). NADA can induce Ca²⁺-influx and calcitonin-gene related peptide (CGRP) and substance-P release in cultured dorsal root ganglion (DRG) neurons (Huang et al., 2002). Moreover, nocifensive behaviors are induced in dosedependent manners when NADA is applied to mouse hind paw. Because NADA possess an arachidonic and dopamine moiety, these precursors were also tested. Equimolar arachidonic or dopamine injections to hind paws were found to ineffectively evoke nocifensive behaviors, suggesting that these precursors do not affect nociceptors. NADA is present in various brain areas, most notably in the striatum of bovine brain. NADA is rarely found in sensory ganglia (DRG) (Huang et al., 2002), and like anandamide, NADA is known to depress certain types of synaptic transmission in the hippocampus (Huang et al., 2002). However, although NADA induces nociceptive behavior when applied to the periphery, its action on peripheral nociception remains unclear. Nevertheless, it is evident that NADA plays a greater role in the CNS, because of its abundance in the brain and its modulatory action on hippocampal synaptic transmission.

C. Metabolic Products of Lipoxygenase

Another type of lipid is known to activate TRPV1. More specifically, 12hydroperoxyeicosatetraenoic acid (12-HPETE) (Fig. 1) and other metabolic products of lipoxygenases (LO) can activate TRPV1 (Hwang et al., 2000). Because capsaicin binds capsaicin channel complex from the intracellular side (Jung et al., 1999), it appears reasonable that endogenous activators are synthesized or released from an intracellular pool (Jung et al., 1999). Thus, intracellular second messengers, such as ATP, AMP, GTP, GMP, or Ca^{2+} , etc, were first tested as potential TRPV1 activators. However, none of these intracellular second messengers activated capsaicin receptors in cultured sensory neurons (unpublished data). However, we found that metabolic products of LOs, namely, 12-HPETE, 15-HPETE, 5-hydroxyeicosatetraenoic acid (5-HETE), 15-HETE, or leukotriene B4 (LTB₄) are able to activate TRPV1 (Hwang et al., 2000). When 12-HPETE and other LO products are applied to a bath of inside-out patches of sensory neurons or HEK cells expressing TRPV1, these metabolites activate single-channel currents in capsazepine-reversible manner. Moreover, the biophysical properties of the channel currents activated by these LO products are identical to those obtained using capsaicin (Hwang et al., 2000). Various LO products have been tested with respect to 12-HPETE channel activation. Of these, 12- and 15-HPETE, 5- and 15-HETE, or LTB₄ were found to be the most potent. 12-HPETE, an immediate metabolic product of 12-LO, activated TRPV1 most potently and effectively with an apparent half-maximal dose (EC_{50}) of 8 μM. Other LO products, such as DiHETE, hepoxilin A3, and LTC₄, were much less effective at activating the TRPV1 (Hwang et al., 2000). Prostaglandins were among the first to be tested for TRPV1 activation because of their wide use as analgesics. To our dismay, none of the prostaglandins were found to activate TRPV1. And, arachidic, linoleic, linolenic, arachidonic acids, and other saturated or unsaturated fatty acids do not or only barely activate TRPV1 (Hwang et al., 2000). It is interesting that among the AA metabolites LO products, but not cyclooxygenase products, activate TRPV1.

D. Binding Capacity of 12-HPETE to TRPV1

Even though it is clear that 12-HPETE and capsaicin act on the same binding sites in TRPV1 because capsazepine blocks 12-HPETE induced currents, it is not known whether 12-HPETE binds TRPV1 directly. To address this issue, Shin and her colleagues performed a competition-binding assay to test the binding of 12-HPETE to TRPV1 (Shin *et al.*, 2002). Because 12-HPETE is not a high-affinity agonist of TRPV1, it is difficult to use it in a

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receptor-binding assay. Thus, we adopted a competition assay approach to examine the binding of 12-HPETE and TRPV1 using 3H-resiniferatoxin (3H-RTX) as a competitor (Acs *et al.*, 1996; Szallasi *et al.*, 1999). As expected, the specific binding of ³H-RTX to TRPV1 was reduced in a dose-dependent manner when various concentrations of 12-HPETE were added. The inhibition constant (K_i) of 12-HPETE in terms of its competing with ³H-RTX binding to TRPV1 was 0.35 μ M, which is lower than that of capsaicin (2.5 μ M) (Shin *et al.*, 2002). Thus, it is evident that 12-HPETE activates TRPV1 by directly binding TRPV1, with a higher affinity than capsaicin (Shin *et al.*, 2002).

E. Comparison of 3D Structures of Capsaicin with 12-HPETE

Because 12-HPETE and capsaicin bind and activate TRPV1, it is logical to assume that the structures of the two chemicals are similar. However, the chemical structures of these two are dissimilar (Fig. 1). It is not easy to envisage how these two structurally-unrelated species act on the same binding site. We postulated that the three-dimensional (3D) structures of the two are similar. To address this issue, the 3D structures of capsaicin and 12-HPETE, 15-HPETE, 5-HETE, LTB₄, and anandamide were 'extracted' by computer modeling. To choose the most stable 3D structures, the lowest energy states were chosen and then the structures were superimposed. Surprisingly, the 3D structure of capsaicin was found to overlap well with that of 12-HPETE (Fig. 2) (Hwang et al., 2000). In earlier studies on capsaicin analogs, the structure of capsaicin was divided into three functional regions, as shown in Fig. 1 (Walpole and Wrigglesworth, 1993). The A-region is an aromatic ring region, the B-region an amide bond region, whereas the C-region is an aliphatic chain region spanning 8-carbon atom (Walpole and Wrigglesworth, 1993). In addition, capsaicin has other functional groups, for example, a phenolic and a methoxyl hydroxide moiety, a dipolar amide group, and the dipolar functional groups were found to be important for hydrogen bonding presumably with capsaicin receptor (Walpole and Wrigglesworth, 1993). When 3D structures were compared, the functional moieties of capsaicin, such as the phenolic hydroxide and amide moieties, were found to overlap well with the functional groups of 12-HPETE, such as its carboxylic acid and hydroperoxide moieties, respectively (Fig. 2). Furthermore, the contour and length of the aliphatic region (C-region) of capsaicin was found to overlap with the alkyl chain of 12-HPETE. Most notably, this result is consistent with what is known about the length of carbon chains in the C-region of active capsaicin analogs. Earlier studies on the structure-activity relationship of capsaicin analogs showed that 8 to 10 carbon atoms represent an optimal



FIGURE 2 Comparison of the 3D structure of capsaicin with those of 12-HPETE, LTB_4 , and anandamide. Reprinted from Hwang and Oh (2002).

alkyl chain length in the C-region (Szolcsanyi and Jancso-Gabor, 1976; Walpole and Wrigglesworth, 1993). Moreover, the structural similarity between capsaicin and 12-HPETE further suggests that they bind and act at the same receptor TRPV1. In addition, 15-HPETE, 5-HETE, LTB₄, and anandamide are much less potent at activating TRV1 than 12-HPETE and show less structural similarity with capsaicin (Fig. 2) (Hwang *et al.*, 2000; Hwang and Oh, 2002). Taken together, 12-HPETE appears to be a good candidate endogenous activator of TRPV1. In line with this notion, 12-HPETE is also known to act on K⁺ channels in *Aplysia* sensory neurons (Piomelli *et al.*, 1987) and in mammalian cardiac muscle cells (Kim *et al.*, 1989).
III. THE TRPV1 LIGAND-BINDING SITES

A. Transmembrane Domain 3 (TM3) Region

The location of agonist binding becomes an important issue when one tries to understand TRPV1 activation mechanisms. After identifying rat TRPV1, Julius and his group cloned a chick ortholog of TRPV1 (Jordt and Julius, 2002). Chick TRPV1 barely responds to capsaicin, although it does respond to acid and heat. Using chimeric constructions of rat and chick TRPV1, they identified a region that accounts for capsaicin sensitivity or vanilloid binding. A small region that spans transmembrane domains 2-4 (TM2-4) of rat TRPV1 (rTRPV1) was found to be sufficient to confer a capsaicin-sensitive current and $[^{3}H]$ -RTX binding. For example, a rat/chick TRPV1 chimera with the chick TRPV1 backbone and the rat TM2-4 segments elicited a robust capsaicin-induced current and [³H]-RTX binding. [³H]-RTX binding was also observed when the TM2-4 segment of rat TRPV1 was replaced with the cognate segment of rat TRPV2 or rat TRPV4. They further narrowed down the minimal segment of TRPV1 that confers capsaicin sensitivity to 45 amino acids. A small segment that in the TM3 region (505~550 aa) appears to be critical for capsaicin sensitive currents and ligand binding, because chick TRPV1 recovers capsaicin sensitivity and ³H]-RTX binding when the TM3 region in chick TRPV1 is replaced by the TM3 region (505~550 aa) in rat TRPV1.

Residues in the transition region between the cytosol and TM3 are conserved among species. Among these conserved residues, the tyrosine and serine residues at 511 and 512 amino acids (Tyr 511 and Ser 512) of rat TRPV1 appear to be important for capsaicin sensitivity and ligand binding (Jordt and Julius, 2002). Point mutations at Tyr 511 and Ser 512 cause selective loss of capsaicin sensitivity to acid and heat to 45 amino acids, respectively. In addition, Jordt and Julius found that the aromatic ring at Tyr 511 is important for vanilloid interaction, because replacement of the Tyr residue with a nonaromatic amino acid caused a complete loss of capsaicin sensitive currents (Jordt and Julius, 2002). With the help of computer modeling of TRPV1, Jordt and Julius further proposed that the aromatic moiety of Tyr 511 interacts with the vanillin moiety of capsaicin, an aromatic–aromatic interaction. A simplified model of binding between TRPV1 and capsaicin is shown in Fig. 3.

The functional significance of the TM3 region in TRPV1 was also observed in rabbit and rat TRPV1 chimeras (Gavva *et al.*, 2004). Like chick TRPV1, rabbit TRPV1 (*Oryctolagus cuniculus*, oTRPV1) does not respond to capsaicin but does respond to acid and heat. When the minimal segment in the TM3 region of rTRPV1 (aa 505–550) was replaced by oTRPV1,



FIGURE 3 (A) A model of capsaic that interacts with the TM3 region and Arg 114 and Glu 761 in the cytosolic tail of TRPV1. (B) Sequence alignment of various orthologs of TRPV1 in the vicinity of Arg 114 and Glu 761.

the r/o TRPV1 chimera elicited capsaicin-sensitive currents (Gavva et al., 2004). These results further confirm the importance of TM3 in vanilloid binding. Gavva and colleagues further narrowed down key amino acid residues in the TM3/4 region that appear to determine sensitivity to RTX. to two residues, Met 547 and Thr 550 in rTRPV1. These two residues appear to be critical for RTX-induced Ca^{2+} influx because rabbit TRPV1 (oTRPV1), which possesses Leu and Ile in these two positions fails to respond to capsaicin or RTX. Moreover, when Leu 547 and Ile 550 in oTRPV1 were replaced by Met and Thr by point mutagenesis, the mutant (oTRPV1-L547M/I550T) recovered RTX-induced Ca²⁺ influx (Gavva et al., 2004). Furthermore, the reverse mutation rTRPV1-M547L failed to show specific binding to RTX. Based on crystal structure of bacterial K⁺ channels, Gavva and colleagues constructed and proposed a model for capsaicinreceptor binding. According to this model, TM3 and TM4 of TRPV1 form a paddle similar to that suggested by McKinnon and colleagues for voltagegated K+ channel (Jiang et al., 2003a,b). This structural model suggests that capsaicin and RTX lie between and interact with the TM3 and TM4 helices. In particular, Gavva and colleagues stressed that the hydroxyl group of Thr 550 would interact with the phenolic hydroxide of the vanilloid moiety of capsaicin or RTX, because this hydroxyl group is capable of hydrogen bonding. According to Gavva's model, the aliphatic region (C-region) of capsaicin probably interacts with the aromatic moiety of Tyr 511. This model disagrees somewhat with the model proposed by Jordt and Julius (2002), who proposed that the aromatic ring region in capsaicin interacts with the aromatic moiety of Tyr 511. At present, it is difficult to determine which model is more accurate. Crystallographic studies will undoubtedly resolve these issues.

B. Ligand-Binding Sites in the N- and C-termini

With the knowledge that capsaicin binds TRPV1 channel from the intracellular side, the search for intracellular ligand-binding sites began with a mutagenesis study (Jung et al., 2002a). Based on the idea that intracellular binding sites would be present in cytosolic tails, Jung and colleagues constructed many deletion mutants at the N- and C-termini of TRPV1 (Jung et al., 2002a). When expressed heterologously in Xenopus oocytes, deletion mutants lose robust capsaicin-sensitive currents if the deletion includes Arg 114 at the N-terminus or Glu 761 at the C-terminus. Moreover, when the two critical sites are omitted by point mutagenesis, these mutants fail to respond to capsaicin with a concomitant loss of specific binding to [³H]-RTX (Jung et al., 2002a). Thus, these two charged amino acids appear to be important for vanilloid binding. Furthermore, the charges of these amino acids are also critical, because their substitution with neutral or oppositely charged amino acids block capsaicin-sensitive currents and specific binding to [³H]-RTX. The functional relevance of these sites for capsaicin sensitivity is also conceivable in vivo, because splicing variants of human TRPV1, which lack a portion of the N-terminus including Arg 114, do not respond to capsaicin (Schumacher et al., 2000). These variants are fairly well expressed in sensory neurons as is TRPV1(Sanchez et al., 2001). Thus, these two sites may be necessary for capsaicin sensitivity, and ligand binding specifically. Since these two sites are charged and located in cytosolic tails, they appear important for hydrophilic interaction with vanilloids, and probably together with the TM3 region comprise the ligand-binding pocket in capsaicin receptor.

Cluster analysis of TRPV1 orthologs of different species revealed that Arg 114 and Glu 761 are highly conserved (Fig. 3B). These two amino acids are also conserved in TRPV1 orthologs that are insensitive to capsaicin, for example, chick TRPV1 (gTRPV1) and rabbit TRPV1 (Jordt and Julius, 2002; Gavva *et al.*, 2004). One might believe think that these two sites are not likely to be important for capsaicin binding because they are also conserved in capsaicin-insensitive orthologs of TRPV1. However, we explain their importance as follows. The TM3 region and the two sites in the N- and C-termini, the minimal triad, constitute the vanilloid-binding pocket, which was suggested earlier (Walpole and Wrigglesworth, 1993). The idea is

consistent with the finding that a mutation in the TM3 region, not in Arg 114 or Glu 761 in the cytosolic tails, causes a lack of capsaicin sensitivity in orthologs of TRPV1 such as in oTRPV1 or gTRPV1. The proposed triad system required for capsaicin sensitivity was also tested in a mutational study. Jung et al. (2002b) constructed a series of TRPV1 chimeras with TRPV2. For these chimeras it was found that as long as the triad remained intact, capsaicin-sensitive channel currents were observed. However, whenever one component of the triad in TRPV1 was replaced by a cognate segment of TRPV2, no capsaicin-sensitive current was observed. TRPV2 lacks the key determinants in TM3 regions and Arg 114 and Glu 761. Thus, this chimera study further suggests that the triad consists of the pharmacologically active ligand-binding sites. Although the triad system appears to account for the ligand-binding pocket of TRPV1, structural insights of capsaicin binding are only conceptual, and crystallographic structural analysis is required. The implications of structural determinants for ligand binding are immense, because they help design potent antagonists in silico for use as analgesics (see in a later section).

IV. NOCICEPTIVE SIGNALS TO TRPV1

A. Bradykinin Signaling Pathway to TRPV1

The associations between 12-HPETE and other 12-LO products and TRPV1 activation, which are implicated in inflammatory pain, beg the question as to which inflammatory signals are linked to TRPV1. When tissues become inflamed, various inflammatory mediators are released from inflammatory cells. Among these inflammatory mediators BK is known to cause pain and pain related reflexes (Reeh and Petho, 2000). BK is released when tissues are inflamed or during other pathological conditions (Dray and Perkins, 1993; Ferreira et al., 1993). Moreover, when BK was injected into rat hind paw, hyperalgesic responses were observed (Bauer *et al.*, 1992; Hong and Abbott, 1994; Ferreira, 2004), and BK is known to excite or sensitize a subset of sensory neurons (Kumazawa et al., 1991, 1996; Michaelis et al., 1998). Despite compelling evidence concerning the excitatory effect of BK, the intracellular signaling mechanisms underlying the effect of BK are poorly understood. In the late 1980s, several groups reported that inositol (1,4,5)-triphosphate and 1,2-diacylglycerol are released as a result of phospholipase C (PLC) activation in sensory neurons after BK application (Thayer et al., 1988; Burgess et al., 1989). In addition to the production of inositol phosphates, BK also increases the level of AA (Thaver et al., 1988; Gammon et al., 1989). Because AA is a substrate of LO, the production of AA by BK leads to the notion that BK might activate TRPV1 via a PLA₂/LO pathway. The application of BK to sensory neurons evokes inward currents (Shin et al., 2002). The whole-cell currents evoked by BK are reduced by cotreating capsazepine (Shin et al., 2002). The activation of TRPV1 by BK is also observed in cell-attached patches in capsazepine-reversible manner, which further suggests that BK activates TRPV1. In an in vitro skin-nerve preparation in which the action potentials of cutaneous C-fibers were recorded (Reeh, 1988; Simone et al., 1989; Steen et al., 1995), BK was found to evoke a volley of action potentials that were reduced by cotreating with capsazepine (Shin *et al.*, 2002). In addition, the excitation of cutaneous nerve fibers by BK was inhibited by nordihydorguaiaretic acid (NDGA), a nonselective inhibitor of LO) and by quinacrine (an inhibitor of PLA₂). The excitatory effect of BK on sensory neurons via LO has been suggested by others. For example, McGuirk and Dolphin (1992) reported that a BK-induced volley of action potentials was attenuated by NDGA (McGuirk and Dolphin, 1992). BK is also known to excite vagal afferent fibers in the airways. In a visceral afferent model, LO inhibitors were found to reduce the BK-induced volley of action potentials in tracheal vagal afferent fibers (Carr et al., 2003). These results indicate that BK excites sensory neurons via the PLA₂/LO/TRPV1 pathway. Even though AA is a substrate of cyclooxygenase, BK does not stimulate cyclooxygenase to excite sensory fibers because the action potential volleys of cutaneous fibers evoked by BK are not blocked by indomethacin, a cyclooxygenase inhibitor (Shin et al., 2002). Because prostaglandins, metabolic products of cyclooxygenase, do not activate TRPV1, it is difficult to consider the cvclooxygenase/TRPV1 pathway for BK signaling. The possible involvement of the LO/TRPV1 pathway as a means of inducing the excitatory effect of BK on sensory neurons was also tested in a Ca²⁺ imaging study using the fluorescent Ca²⁺ sensitive dye, Fluoro-3 AM. Portions of sensory neurons fluoresce green in response to BK application after incubation with Fluoro-3 AM. As in skin-nerve preparations, the Ca² ⁺ influx induced by BK was greatly reduced by capsazepine, quinacrine, NDGA, or baicalein (a 12-LO specific inhibitor) (Shin et al., 2002). Even though electrophysiological and Ca^{2+} imaging experiments suggest the participation of an intracellular signaling pathway in BK response, the actual synthesis of LO metabolic products after BK application needs to be presented in sensory neurons in order to confirm the involvement of this signaling pathway. More than a tenfold increase in 12-HETE, an immediate metabolic product of 12-HPETE, was observed in sensory neurons after BK application (Shin et al., 2002). Consistent with this, a two fold increase in the level of LTB₄, a 5-LO metabolic product, was observed after BK injection into the mouse hind paw (Ferreira et al., 2004). Thus, it is now accepted that BK excites sensory neurons by stimulating the PLA₂/LO/TRPV1 pathway, as shown in Fig. 4.



FIGURE 4 Proposed BK signaling pathways for sensory neuron excitation.

This proposed BK signaling pathway has been proven in part by *in vivo* experiments (Shin *et al.*, 2002; Ferreira *et al.*, 2004). Initially, Levine's group reported that LO products such as LTB_4 induce hyperalgesia when injected intradermally (Levine *et al.*, 1984; Martin *et al.*, 1988), whereas Shin *et al.* found that LO inhibition reduces the thermal hyperalgesia induced by BK (Shin *et al.*, 2002). Ferreira *et al.* (2004) found that an intradermal injection of BK causes an overt nociceptive response, and that co-injection of capsazepine with BK almost completely abolished BK-induced nocifensive response. Consistent with this, nociceptive BK response was almost abrogated when hind paws were desensitized by capsaicin treatment. These results further confirm that BK uses the PLA₂/LO/TRPV1 pathway to excite sensory neurons *in vivo*. However, a PKC inhibitor was found to reduce BK-induced paw licking (Ferreira *et al.*, 2004), and therefore, the possibility that other signaling pathways, such as the PKC pathway, mediate the effect of BK *in vivo* cannot be ruled out.

B. 20-HETE Action on TRPV1

Some reports indicate that another eicosanoid, 20-HETE, a metabolic product of cytochrome P-450 (Fleming, 2001; McGiff and Quilley, 2001; Roman, 2002), activates TRPV1. In addition to cyclooxygenase and LOs, cytochrome P-450 also metabolizes AA to yield 20-HETE and epoxyeicosa-trienoic acids. 20-HETE is known to be released by vascular smooth muscles (Imig *et al.*, 1996). It is a potent vasoconstrictor in renal arterioles and in other

5. Nociceptive Signals to TRPV1 and its Clinical Potential

smooth muscles (Imig *et al.*, 1996), and in guinea pig airways 20-HETE constricts smooth muscles (Rousseau *et al.*, 2005). Moreover, 20-HETE induced vasoconstriction was reduced by capsazepine dose-dependently, suggesting that 20-HETE induces vasoconstriction by activating TRPV1 (Rousseau *et al.*, 2005). Similarly, 20-HETE constricts mesenteric resistant arteries (Scotland *et al.*, 2004), and this is greatly reduced by capsazepine (Scotland *et al.*, 2004). The involvement of the 20-HETE/TRPV1 pathway has been suggested to explain the Bayliss' myogenic reflex [the constriction of resistant arteries in response to intraluminal pressure (Scotland *et al.*, 2004)] of arteries. Even though direct evidence that 20-HETE (a product of cytochrome P-450) activates TRPV1 is lacking, substantial evidence indicates a pathophysiological role of the 20-HETE/TRPV1 pathway in vascular smooth muscle contraction.

C. Histamine Intracellular Signals in Sensory Neurons

It might be considered that other inflammatory mediators would use the same PLA2/LO/TRPV1 pathway as BK. One such candidate is histamine, which is released from mast cells after tissue injury, and is known to cause itching (Magerl et al., 1990; Ward et al., 1996; Stander et al., 2003). Moreover, high levels of histamine are present in the skins of atopic dermatitis patients (Ring, 1983; Ruzicka and Gluck, 1983). Histamine excites a subgroup of sensory neurons (Magerl et al., 1990; Ward et al., 1996; Koppert et al., 2001; Lischetzki et al., 2001), especially mechanical insensitive C-fibers (Schmelz et al., 2003). Even though the effects of histamine on sensory neurons are well documented, the mechanisms underlying the excitatory effect of histamine are not well understood. There appears to be a casual relationship between TRPV1 and histamine response, because histamine excites a subgroup of neurons that respond to capsaicin, even though it was reported that a subpopulation of histamine-sensitive sensory neurons are not sensitive to capsaicin (Handwerker et al., 1991; Schmelz et al., 1997, 2003; Nicolson et al., 2002). In addition, histamine was reported to release AA via the action of PLA₂ in Chinese hamster ovary cells (Leurs et al., 1994). Thus, it might be hypothesized that histamine excites sensory neurons via the PLA₂/LO/TRPV1 pathway. To test the notion of sensory neurons excitation by histamine, we measured the Ca^{2+} influx induced by histamine treatment in cultured sensory neurons using the Ca²⁺ sensitive fluorescent dye, Fluo-3/AM (Kim *et al.*, 2004). Histamine application evoked a transient increase of intracellular Ca^{2+} in a dose-dependent manner via H1-receptor. Moreover, histamine-induced [Ca²⁺]_i increase was reversibly inhibited by



FIGURE 5 Proposed signaling pathway for the excitation of sensory neuron by histamine, a pruritogenic substance.

capsazepine or SC0030 (another competitive antagonist of TRPV1), suggesting the mediation of TRPV1 in histamine-induced Ca^{2+} influx (Kim *et al.*, 2004). Treatment of quinacrine and NDGA markedly inhibited histamineinduced $[Ca^{2+}]_i$ in dorsal root ganglion neurons (Kim *et al.*, 2004). Thus, it appears likely that histamine activates TRPV1 via the PLA₂/LO pathway in order to excite sensory neurons (Fig. 5).

Because BK and histamine use the same PLA₂/LO/TRPV1 pathway in sensory neurons, we propose that this signaling pathway serves as a common pathway for the mediation of inflammatory signals and for the excitation of sensory neurons. However, this proposition remains to be investigated. Moreover, histamine-sensitive C-mechano insensitive neurons also respond to capsaicin and BK (Lang et al., 1990; Schmelz et al., 2003). Thus, in some cases, BK and histamine are likely to use the PLA₂/LO/TRPV1 pathway at the same sensory neurons. Then the question should be asked as how a given sensory neuron responds to both algogenic and pruritogenic substances. One possible explanation is that this type of sensory neuron would mediate itch and pain. In fact, injected histamine sometimes induces a "burning" pain rather than an itch (Keele and Armstrong, 1964; Koppert et al., 1993; Schmelz et al., 2003). Another explanation would be that itch and pain are controlled and determined by central pathways. In addition, some BKsensitive sensory fibers do not respond to histamine (Lang et al., 1990), which suggests that histamine "specific" fibers conduct itch signals to the brain. In this case, BK and histamine would use the PLA₂/LO/TRPV1 pathway in different sets of sensory neurons.

Whatever the roles of histamine-sensitive neurons are, it is likely that histamine uses the PLA₂/LO/TRPV1 pathway to excite sensory neurons. However, we cannot exclude the possibility that BK or histamine also excites sensory neurons after TRPV1 has been sensitized by various kinases or

PLC. These specific sensitization signal mechanisms are reviewed in the Chapters 4 and 6.

V. TRPV1 ANTAGONISTS: A NEW CLASS OF ANALGESICS

As TRPV1 is believed to be an important factor for the generation of nociceptive neural signals, it has become a target for the development of a novel class of analgesics. Many pharmaceutical companies have already developed series of compounds that have strong antagonistic activity to TRPV1 and analgesic effects *in vivo*. In this chapter, we introduce some of the newly synthesized TRPV1 antagonists.

A. Capsazepine

Capsazepine was the first TRPV1 antagonist to be synthesized based on the capsaicin structural backbone (Fig. 6) (Walpole and Wrigglesworth, 1993; Walpole *et al.*, 1994). Capsazepine was synthesized as a competitive and selective antagonist for capsaicin receptor and has been used as a pharmacological tool to identify native capsaicin receptor in sensory neurons or TRPV1. Moreover, its availability has enabled enormous progress in the study of TRPV1 and related fields. Walpole and Wrigglesworth (1993) synthesized numerous capsaicin receptor agonists and came to the conclusion that the phenolic hydroxyl and amide bond of capsaicin (Fig. 6) are required for agonistic activity. They further found that when the A- and



FIGURE 6 TRPV1 antagonists (I).

B-regions in capsaicin (Fig. 1) were arranged in a coplanar manner, agonistic activity was much enhanced. After making a series of analogs in which the two regions were constrained, Walpole and Wrigglesworth found that a sevenmembered ring analog, capsazepine (Fig. 6), showed potent antagonistic activity but no agonistic activity. Capsazepine was the first competitive and selective antagonist of moderate potency (IC₅₀ = $\sim 0.5 \,\mu$ M) (Walpole and Wrigglesworth, 1993). Even though capsazepine has been useful in *in vitro* experiments as a competitive antagonist, little information is available on its effects in vivo. One reason for this is that capsazepine is highly insoluble in water (Kwak et al., 1998). Moreover, because capsazepine is a capsaicin analog, it is likely that capsazepine has the poor metabolic and pharmacokinetic properties common to capsaicin analogs (Wehmeyer et al., 1990). Another factor that hampers the use of capsazepine as a target compound for clinical use is its relative nonselectiveness. Capsazepine inhibits acetylcholine receptors (Liu and Simon, 1997), HCN1 channel (Gill et al., 2004), and voltage-gated Ca²⁺ channel (Docherty et al., 1997). Thus, from the pharmaceutical point of view, capsazepine is not favorably viewed because of its apparent shortcomings.

B. SC0030

SC0030 is another TRPV1 antagonist synthesized based on the capsaicin and 12-HPETE structural backbones. Suh et al. (2003) synthesized a series of compounds whose structures were based on capsaicin and capsazepine. On analyzing the structural requirements for TRPV1 antagonistic activity, they found that the thiourea in the B-region of capsaicin is critical for agonistic and antagonistic activity and that replacing the methoxy of the vanilloid moiety of capsaicin with fluoride elicited strong antagonistic activity as a hydrogen bond acceptor (Fig. 6). When in addition to this replacement, the hydroxy group in the vanilloid moiety was replaced with methansufonamide as a hydrogen bond donor, the resulting analog, SC0030, was found to possess potent TRPV1 antagonistic activity (Suh et al., 2003). In Ca²⁺ influx testing, the IC₅₀ of SC0030 in terms of antagonizing capsaicin-induced Ca^{2+} influx was about 0.037 μ M (Suh *et al.*, 2003). The antagonistic pharmacological activities of SC0030 were also determined by Wang and his colleagues (Wang et al., 2002;see also Erratum Wang et al., 2003)¹. SC0030 inhibits [³H]-RTX binding with a K_i of 0.053 μ M and antagonizes capsaicin-induced Ca^{2+} uptake with an IC₅₀ of 9.2 nM (Wang

¹SC0030 was erroneously dubbed as JYL1421 by one of co-authors and described as if he synthesized it (see for corrected version) (Wang *et al.*, 2003).

et al., 2002). SC0030 is also known to inhibit acid and heat-induced Ca^{2+} influx in TRPV1 expressing cells (Wang *et al.*, 2002).

C. Iodo-Resiniferatoxin

RTX is a product of Euphorbia resinifera (a cactus-like plant) and an extremely irritant substance (Szallasi and Blumberg, 1999). RTX is now known to be a potent agonist of TRPV1, and because of this it has been used as a pharmacological tool for assaying the binding abilities of ligands and antagonists (Szallasi and Blumberg, 1990, 1999; Szallasi et al., 1994). Because RTX is so potent, it would be logical to use the RTX backbone in order to design strong antagonists. Wahl et al. (2001) synthesized iodo-RTX (I-RTX) as a TRPV1 antagonist. They attached iodine to the 5-carbon position in RTX (Fig. 6). As expected for a RTX analog, I-RTX was found to have strong antagonistic activity and inhibited capsaicin-induced currents at the nanomolar level (IC₅₀ = $0.69 \sim 5.4$ nM) (Seabrook *et al.*, 2002). I-RTX is a competitive antagonist for TRPV1 because its radiolabeled analog, $[^{125}I]$ -RTX, binds TRPV1 in a reversible manner with an apparent K_D at 4.3 nM, which is twice as potent as capsazepine (Wahl et al., 2001). In addition, I-RTX inhibits heat-activated and acid activated currents almost completely (Seabrook et al., 2002), in contrast to the incomplete antagonistic action of capsazepine with respect to acid-induced current responses (Seabrook et al., 2002). However, although I-RTX shows strong antagonistic activity in vitro, it does not block capsaicin-induced pain behavior (flinching) in vivo. Instead, I-RTX induces a TRPV1-independent irritation when injected into rat hind paw (Seabrook et al., 2002). Answers for the discrepancies between the actions of I-RTX in vivo and in vitro require further study.

D. High-Throughput Screening for TRPV1 Antagonists

High-throughput screening (HTS) is an efficient and rapid way of testing a wide variety of chemical libraries for active lead compounds. After cloning TRPV1, many pharmaceutical companies adopted this technique to search for active lead compounds that antagonize TRPV1. As explained by Gunthorpe and colleagues (Gunthorpe *et al.*, 2004), cells stably expressing TRPV1 are plated on 96- or 384-well plates, which are then incubated with Ca^{2+} -sensitive fluorescent dye such as Fluoro-3/AM. After washing out the fluorescent dye, cells in plates are loaded with different compounds and this is followed by measuring changes in fluorescent intensity using a

fluorescence imaging plate reader (FLIPR). This technique is an efficient means of identifying active compounds that antagonize capsaicin-induced Ca^{2+} influx in TRPV1-expressing cells among a large number of compounds in chemical libraries. Another advantage of the HTS system is that it allows for searches of active chemicals among structurally diverse compounds that do not contain the capsaicin pharmacopore. Although HTS technique is useful for saving time for the screening of large numbers of compounds, it has disadvantages. The use of HTS often leads to similar findings in different laboratories, thus several different laboratories or companies end up working on the same active compounds. The search for TRPV1 antagonists using HTS was no exception, as explained in the following section.

E. SB-366791, AMG9810, and their Analogs

Gunthorpe and colleagues developed SB-366791 as a TRPV1 antagonist using a HTS technique (Gunthorpe et al., 2004). SB-366791 has a methoxyphenyl acrylamide moiety that appears to be structurally similar to that of capsaicin, because this moiety has an aromatic ring and an amide (A and B) region (Fig. 6). SB-366791 is a competitive antagonist that antagonizes capsaicin-induced Ca^{2+} influx and capsaicin-induced current with an IC₅₀ of 5.7~7.5 nM (Gunthorpe et al., 2004). SB-366791 also inhibits acid- and heat-induced currents. Unlike capsazepine, SB-366791 is relatively specific for TRPV1 and does not affect the activities of HCN channel or voltagegated Ca^{2+} currents in rat sensory neurons (Gunthorpe *et al.*, 2004). A team in Amgen in USA also synthesized TRPV1 antagonists that were structurally related to SB-366791. AMG9810 is a competitive antagonist of TRPV1 that has an acrylamide core (Fig. 7) (Doherty et al., 2005; Gavva et al., 2005). Amgen later optimized AMG9810 further to synthesize more potent analogs. Some analogs of AMG9810 (lower panel in Fig. 7) show TRPV1 antagonistic activity with IC50s of less than 1 nM in terms of inhibiting capsaicin- and acid-induced Ca^{2+} influx, with much greater bioavailability (17~39%) (Doherty et al., 2005).

F. BCTC and its Analogs

Pyridinyl piperazine urea analogs have been identified as strong TRPV1 antagonists by many pharmaceutical companies. One of the leading compounds is BCTC [*N*-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2H)-carboxamide] (Fig. 7) (Sun *et al.*, 2003; Valenzano *et al.*, 2003). BCTC elicits antagonistic activity with an IC₅₀ of \sim 35 nM to

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FIGURE 7 TRPV1 antagonists (II).

capsaicin-induced Ca²⁺ influx in HEK cells that stably express rat TRPV1. BCTC also antagonizes acid induced Ca^{2+} influx with an IC₅₀ of 6 nM. Moreover, BCTC is specific to TRPV1 because no major interactions were observed when it was tested for binding to 62 different receptors (Sun et al., 2003; Valenzano et al., 2003). Furthermore, BCTC is effective at inhibiting capsaicin or inflammation-induced pain-related behaviors in vivo, and BCTC reverses capsaicin-induced hyperalgesia in rats. Moreover, oral injection of BCTC significantly inhibits thermal hyperalgesia and mechanical hyperalgesia caused by capsaicin (Pomonis et al., 2003). Orally available BCTC also reduces inflammation-associated thermal and mechanical hyperalgesia in rats in a manner comparable to that of indomethacin. Most surprisingly, the oral administration of BCTC reduced neuropathic pain. In a partial sciatic nerveligation model, oral BCTC was found to significantly reverse mechanical hyperalgesia and allodynia induced by nerve injury, in a manner similar to gabapentin (Pomonis et al., 2003). It was also shown recently that an analog of BCTC is effective at reducing some forms of cancer pain (Ghilardi et al., 2005). Thus, BCTC and possibly other TRPV1 antagonists could be used in a clinical setting as potential treatments for some types of cancer or neuropathic pain.

However, BCTC has pharmacologic limitations because it has a short half-life and poor metabolic stability or low bioavailability (Tafesse *et al.*, 2004). In addition, BCTC inhibits human ether-a-go-go (HERG) channel activity by \sim 80%, which could prolong the cardiac QT interval and cause ventricular arrhythmia and fibrillation (Tafesse *et al.*, 2004). More recently, Tafesse and colleagues synthesized a new series of pyridinyl piperazine compounds with improved pharmacological and pharmacokinetic properties

and low HERG inhibition (Tafesse *et al.*, 2004). Swanson *et al.* (2005) in Johnson and Johnson Co. also developed a new class of pyridinyl piperazine compounds as TRPV1 antagonists (Fig. 7). Using a matrix synthesis approach that targeted changing functional groups in pyridine, piperazine and aromatic ring regions, and the urea linkage in BCTC, the Johnson and Johnson group came up with a selective and potent TRPV1 antagonist in terms of antagonizing the effect of capsaicin on human TRPV1 (IC₅₀ = 74 nM) (Swanson *et al.*, 2005). Like BCTC, this compound also antagonizes capsaicin-induced behavioral responses such as the tactile allodynia and thermal hyperalgesia and hypothermia induced by capsaicin (Swanson *et al.*, 2005).

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CHAPTER 6

Gating, Sensitization, and Desensitization of TRPV1

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I. OVERVIEW

Single-channel currents of TRPV1 evoked by three different stimuli (capsaicin, proton, or heat) suggest that TRPV1 channel is gated directly by the stimuli. Extensive analyses in the single-channel and whole-cell level reveal not only the detailed kinetics of TRPV1 channel but also voltage- and timedependent properties and amino acids involved in channel activation. Furthermore, it became clear that TRPV1 can form homo-multimers without other subunits in native neurons from the comparison between cloned and native channel properties. TRPV1 can be sensitized through different mechanisms including phosphorylation by several kinases such as PKA and PKC. Desensitization also occurs through several mechanisms including phosphorylation/dephosphorylation and calmodulin (CaM)-mediated one. Membrane-derived lipids regulate TRPV1 function as well. Thus, regulation mechanism of TRPV1 is very complicated. However, given that fact TRPV1 is a key molecule in peripheral nociception, modulation of TRPV1 function will lead to the development of novel antinociceptive or anti-inflammatory agents.

II. INTRODUCTION

Hot chili peppers have been for long time used in food, and people know well that the peppers produce a burning sensation in their mouth. Data obtained using electrophysiological methods provided evidence that vanilloid receptor activation allows cation influx through its ionic pore, leading to the depolarization of the nociceptive neurons, followed by action potential generation. This seems to be one of the mechanisms in which noxious stimuli are converted to the electric signals, which are then transmitted to the brain through the ascending sensory pathways, leading to the perception of pain. At the same time, Ca^{2+} influx through the vanilloid receptor in the nociceptive neuron endings was found to cause the release of some substances, such as substance P (SP) and calcitonin gene-related peptide (CGRP), a phenomenon called "neurogenic inflammation." High Ca²⁺ permeability of the vanilloid receptor (Szallasi and Blumberg, 1999) allowed Julius and colleagues to isolate the gene encoding the capsaicin receptor by using a Ca²⁺ imaging-based expression cloning strategy in 1997 (Caterina et al., 1997). The 838 amino acid proteins encoded by the cDNA was designated vanilloid receptor 1 (VR1) and later renamed TRPV1 as the first member of the transient receptor potential vanilloid (TRPV) subfamily of large TRP super family of ion channel. From its deduced amino acid sequence, TRPV1 was predicted to function as an ion channel with six transmembrane (TM) domains, a pore-loop domain, and cytosolic amino and carboxyl termini.

On exposure to capsaicin, TRPV1 exhibits an outwardly rectifying, nonselective cation current with high Ca^{2+} permeability (Caterina *et al.*, 1997). In addition, TRPV1 was found to be activated not only by capsaicin but also by proton and heat (>43 °C) all of which are known to cause pain *in vivo* (Tominaga *et al.*, 1998). Information regarding gating, sensitization, and desensitization of TRPV1, therefore, could prove useful for the development

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of novel antinociceptive or anti-inflammatory agents since TRPV1 plays a pivotal role in nociception.

III. GATING PROPERTIES OF TRPV1

Clear single-channel currents can be observed on application of capsaicin, proton, or heat in the patches excised from human embryonic kidneyderived HEK293 cells heterologously expressing TRPV1 (Fig. 1), suggesting the direct gating of TRPV1 by the three different stimuli.

A. Capsaicin Action

Because capsaicin and its analogs, such as resiniferatoxin (RTX), are lipophilic, it is quite possible that they pass through the cell membrane and act on binding sites present in the intracellular surface of TRPV1. Indeed, the existence of capsaicin-binding sites in the cytosolic domain of TRPV1 was proved using a synthetic water-soluble capsaicin analog (Jung *et al.*, 1999). Capsaicin is structurally related to putative endogenous TRPV1 agonists such as palmitoyl ethanolamide (anandamide) (Zygmunt *et al.*, 1999), 12-hydroperoxyeicosatetraenoic acid (12-HPETE) (Hwang *et al.*, 2000), and *N*-arachidonoyl dopamine (NADA) (Huang *et al.*, 2002). Therefore, there is significant pharmacological and physiological interest in identifying regions of the channel that transduce the effects of these molecules. Especially, the fact that the three-dimensional (3D) structures of capsaicin and 12-HPETE can be superimposed in the energy-minimized state suggests that action sites of capsaicin is not a pin-point amino acid (Hwang *et al.*,



FIGURE 1 Single-channel recordings of TRPV1 activated by capsaicin (cap, 100 nM), heat ($44 \circ C$), or protons (pH 5.4) at +40 mV in inside/out (cap and heat) or outside/out (protons) configurations. Broken lines indicate closed-channel level. Modified from Tominaga *et al.* (1998).



FIGURE 2 Amino acids involved in activation and permeation of TRPV1. A, ankyrin repeat domain.

2000). Comparison of rat TRPV1 with its avian ortholog from chicken sensory neurons together with mutational analysis revealed that Y511 and S512, located at the transition between the second intracellular loop and the third TM domain, might interact with vanilloid ligands at the intracellular face of the membrane (Fig. 2) (Jordt and Julius, 2002). In addition, R114 and E761 in the amino and carboxyl termini, respectively, were found to be involved in agonist recognition, based on studies involving stepwise deletions of TRPV1 and chimera construction between TRPV1 and its capsaicin-insensitive homolog, TRPV2 (VRL-1) (Fig. 2) (Jung *et al.*, 2002). The apparently wide distribution of residues necessary for capsaicin binding is consistent with the fact that TRPV1 can be activated by compounds, such as capsaicin and 12-HPETE, that are related to one another in their 3D structures and also suggests that these critical residues are relatively close to each other in native channel.

TRPV1 shares structural similarity with the voltage-gated K^+ channels, including six TM topologies (Fig. 2). According to the current helix-packing models of the voltage-gated K^+ channels, derived from helical periodicity analysis and crystallographic approaches, the first, second, and third TM domains are located on the lipid-facing periphery of the tetrameric channel complex, whereas the fifth and sixth TM domains are located closer to the pore-forming channel core. Assuming similar helix packing for TRPV1, the lipophilic moiety of capsaicin may bind to the second and third TM domains on the channel-lipid interface, while the vanilloid moiety may interact with residues around Y511 in the cytosolic region, thus linking the two TM domains together with cytosolic tail.

Single-channel conductance of capsaicin-activated currents for K⁺ or Na⁺ was reported to be 76–105 pS at positive potentials (+60 mV) and 35–46 pS at negative potentials (-60 mV) in HEK293 cells (Caterina *et al.*, 1997; Premkumar *et al.*, 2002) and 80–101 pS (+60 mV) and 43–51 pS (-60

mV) in DRG neurons (Oh *et al.*, 1996; Premkumar *et al.*, 2002), indicating the outwardly rectifying current–voltage (I-V) relationship. At positive potentials, the channel exhibits a single conductance state, whereas brief subconductance states are apparent. The probability of the channel being open (P_o) is also similar between cloned and native TRPV1-mediated currents and is dependent on the TM voltage; greater at positive potentials than at negative potentials (Premkumar *et al.*, 2002). These findings indicate that TRPV1 can form homo-multimers without other subunits in native neurons and that the outward rectification observed in whole-cell currents is due to the voltage dependence of single-channel conductance and P_o .

B. Proton Action

Acidification of the extracellular milieu has two primary effects on TRPV1 function. First, extracellular protons increase the potency of heat or capsaicin as TRPV1 agonists, in part, by lowering the threshold for channel activation by either stimulus. Second, extracellular protons can, themselves, be viewed as agonists because further acidification (to pH < 6.0) leads to channel opening at room temperature (Tominaga et al., 1998). Extracellular protons are believed to act primarily by increasing P_0 (Tominaga *et al.*, 1998; Baumann and Martenson, 2000) rather than by altering unitary conductance or interacting directly with the vanilloid-binding site, although protons reduce the amplitude of the unitary currents a little bit (Baumann and Martenson, 2000). Acidic solution evoked ionic currents with a EC₅₀ value of about pH 5.4 when applied to outside-out but not inside-out membrane patches excised from HEK293 cells expressing TRPV1 (Tominaga et al., 1998), suggesting that protons act on amino acids in the extracellular domain of TRPV1 having side chain pKa values in the physiologically relevant range. Mutational analyses revealed that E600, located within a putative extracellular domain, serves as an important regulator site for proton potentiation of TRPV1 activity, whereas E648 is involved in direct proton-evoked activation of TRPV1 (Fig. 2) (Jordt et al., 2000). These data indicate the existence of stimulus-specific steps in the TRPV1 activation process. A stimulus-specific gating mechanism is also supported by the existence of mutants exhibiting stimulus-specific reduction of their activity (Welch et al., 2000; Kuzhikandathil et al., 2001). In addition to its activating or modulating effects on TRPV1, protons were found to permeate the nonselective TRPV1 pore in acidic extracellular solution, resulting in a marked intracellular acidification (Hellwig et al., 2004). This phenomenon suggests a proton hopping permeation mechanism along with the water-filled channel pore.

C. Heat Activation

Heat-evoked TRPV1 currents show properties similar to those of capsaicinevoked currents. Heat-evoked single channel openings were observed in inside-out membrane patches excised from HEK293 cells expressing TRPV1, suggesting that TRPV1 is itself a heat sensor. It is not clear how and where heat acts to open the TRPV1 channel. However, it is now known that several TRP family ion channels (TRPV1, TRPV2, TRPV3, TRPV4, TRPM8, TRPA1) are thermosensitive, suggesting that temperature sensor domains are present in these channel proteins (Jordt et al., 2003; Patapoutian et al., 2003; Tominaga and Caterina, 2004). The distal half of the TRPV1 carboxyl terminus was reported to be partially involved in thermal sensitivity (Vlachova et al., 2003). Furthermore, the facts that certain mutations and phosphorylation by PKC lead to the reduction of the threshold temperature for TRPV1 activation (Tominaga et al., 2001; Numazaki et al., 2002) suggest more global effects of heat on TRPV1. TRPV1 is known to have a voltage-dependent gating property as described later (Gunthorpe et al., 2000). It was reported that temperature sensing in TRPV1 and TRPM8 (activated by cold stimulus and menthol) is tightly linked to voltage-dependent gating (Voets et al., 2004). TRPV1 is activated upon depolarization, and changes in temperature result in graded shifts in its voltage-dependent activation curve. This result suggests that amino acids responsible for voltage dependence are also involved in thermosensing, although the fourth TM domain of TRPV1 lacks the multiple positively charged residues typical of voltage-gated channels.

TRPV1 currents observed at whole-cell level have been reported to have relatively high permeability for divalent cations ($P_{Ca/Na} = 9.6$ and $P_{Mg/Na} = 5.0$) (Caterina *et al.*, 1997). On the other hand, heat-evoked TRPV1 currents show a similar preference for divalent cations, albite with a little different relative permeabilities ($P_{Ca/Na} = 3.8$ and $P_{Mg/Na} = 3.1$) (Tominaga *et al.*, 1998), suggesting the different gating mechanisms between capsaicin and heat.

D. Voltage- and Time-Dependent Properties

I-V relationships at a whole-cell configuration have reversal potentials close to 0 mV indicating the opening of nonselective cationic channel and exhibit substantial outward rectification with a region of negative slope conductance at negative to around -70 mV (Gunthorpe *et al.*, 2000). The open-time distributions of single-channel currents in the presence of low-capsaicin concentrations (10–30 nM) are best fitted with three exponential components, while the closed-time distributions are best fitted by five

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exponential components, although number of exponential components seems to be different at higher-capsaicin concentrations $(0.5-1 \ \mu\text{M})$ (Premkumar *et al.*, 2002). These single-channel properties suggest the complex kinetics of TRPV1 channel gating. However, the single-channel properties show no significant differences between the native (sensory neurons) and cloned receptors, indicating again that native functional TRPV1 channel is made up of TRPV1 subunit.

E. Permeability

The region important for the cation permeability of TRPV1 has not been well defined. Replacement of D646, an amino acid that is involved in changing ligand affinity (Welch *et al.*, 2000), with N was found to decrease tenfold ruthenium red blockade efficacy and reduce fourfold the relative permeability of the divalent cation Mg^{2+} with respect to Na⁺ without changing the selectivity of monovalent cations (Fig. 2) (Garcia-Martinez *et al.*, 2000). It has also been reported that Y671 in the sixth TM domain is important for Ca²⁺ permeability as described later (Fig. 2) (Mohapatra *et al.*, 2003).

IV. SENSITIZATION OF TRPV1

Inflammatory pain is initiated by tissue damage/inflammation and is characterized by hypersensitivity both at the site of damage and in adjacent tissue. Stimuli that normally would not produce pain do so (allodynia), while previously noxious stimuli evoke even greater pain responses (hyperalgesia). One mechanism underlying these phenomena is the sensitization of ion channels such as TRPV1. Sensitization is triggered by extracellular inflammatory mediators that are released *in vivo* from surrounding damaged or inflamed tissues and from nociceptive neurons themselves (i.e., neurogenic inflammation) (Woolf and Salter, 2000; Julius and Basbaum, 2001). Mediators known to cause sensitization include prostaglandins, adenosine, serotonin, bradykinin, and ATP (Numazaki and Tominaga, 2004). Tissue acidification is also induced in the context of inflammation as described previously.

A. Phosphorylation by PKA

Like other ion channels, TRPV1 can be phosphorylated by several kinases including PKA (De Petrocellis *et al.*, 2001; Bhave *et al.*, 2002; Hu *et al.*, 2002;



FIGURE 3 Proposed mechanisms involved in GPCR-mediated sensitization of TRPV1. G_q-coupled receptor activation leads to production of IP₃ and DAG from PIP₂ through phospholipase C (PLC). PKC activation by DAG causes phosphorylation of TRPV1 (P), leading to functional potentiation (+). PKA and protons sensitizes TRPV1 as well. PIP₂ inhibits TRPV1 (-). PKC activates PLA₂ leading to the AA production, followed by generation of LOX-derived products, which in turn activate TRPV1. Modified from Numazaki and Tominaga (2004).

Rathee *et al.*, 2002), PKC (Premkumar and Ahern, 2000; Tominaga *et al.*, 2001; Sugiura *et al.*, 2002; Bhave *et al.*, 2003), Ca²⁺/CaM-dependent kinase II (CaM kinase II) (Jung *et al.*, 2004), and Src kinase (Jin *et al.*, 2004). There has been extensive work demonstrating that activation of a PKA-dependent pathway by inflammatory mediators, such as prostaglandins, influences capsaicinor heat-mediated actions in sensory neurons, probably by acting on TRPV1 (Fig. 3). These results suggest that PKA plays a pivotal role in the development of hyperalgesia and inflammation by inflammatory mediators. S116 and T370 in the amino terminus were reported to be phosphorylated by PKA and involved in desensitization (Fig. 4) (Bhave *et al.*, 2002; Mohapatra and Nau, 2003). Phosphorylation of S116 by PKA was found to inhibit dephosphorylation of TRPV1 caused by capsaicin exposure. T144, T370, and S502 were also found to be involved in sensitization (Fig. 3) (Rathee *et al.*, 2002).

B. Phosphorylation by PKC

PKC-dependent phosphorylation of TRPV1 occurs downstream of activation of G_q -coupled receptors (GPCRs) by several inflammatory mediators including ATP, bradykinin, serotonin, prostaglandins, and trypsin or tryptase (Fig. 3) (Premkumar and Ahern, 2000; Tominaga *et al.*, 2001; Sugiura *et al.*, 2002, 2004; Moriyama *et al.*, 2003; Amadesi *et al.*, 2004; Dai *e*



FIGURE 4 Regions and amino acids involved in TRPV1 sensitization and desensitization. PIP_2 binds to the indicated region in the carboxyl (C) terminus. CaM binds to both C and N (amino) termini (the first ankyrin repeat; A). PKA, PKC, or CaMKII phoshorylates overlapping serine (S) or threonine (T) residues indicated by arrows. Modified from Tominaga (2005).

2004; Moriyama et al., 2005). PKC-dependent phosphorylation of TRPV1 caused not only potentiation of capsaicin- or proton-evoked responses but also reduced the temperature threshold for TRPV1 activation so that normally nonpainful temperatures in the range of normal body temperature were capable of activating TRPV1, thereby leading to the sensation of pain (Fig. 5). These phenomena were also confirmed in native sensory neurons. Capsaicin potency was modulated by PKC-dependent phosphorylation, suggesting the interaction between phosphorylation and capsaicin-binding. Direct phosphorylation of TRPV1 by PKC was proved using a biochemical approach (Numazaki et al., 2002), and two target serine residues (S502 and S800) were identified (Fig. 4) (Numazaki et al., 2002; Bhave et al., 2003). In the mutant where the two serine residues were replaced with alanine, sensitization or potentiation of TRPV1 activity induced by any of three different stimuli (capsaicin, proton, or heat) was abolished. S502 and S800 were also found to be involved in potentiation of NADA-induced TRPV1 activation (Premkumar et al., 2004), oleoylethanolamide (OEA)-induced TRPV1 activation (Ahern, 2003), and rephosphorylation of TRPV1 after Ca²⁺dependent desensitization (Mandadi et al., 2004).

Both PKA- and PKC-dependent pathways have been reported to function upon same ligands such as serotonin or prostaglandins (Sugiuar *et al.*, 2004; Moriyama *et al.*, 2005). The physiological relevance of the two different pathways downstream of serotonin or prostaglandin exposure remains to be elucidated. The fact that only PKC activation leads to the reduction of temperature threshold for TRPV1 activation might be pertinent to this issue (Moriyama *et al.*, 2005). Disruption of interaction between phosphatidylinositol-4,5bisphosphate (PIP₂) and TRPV1 has also been reported to be involved



FIGURE 5 Reduction of temperature threshold for TRPV1 activation by prostaglandin E_2 (PGE₂, I μ M) in HEK293 cells expressing TRPV1 and EP₁. Representative temperature response profiles in the absence (upper) and presence (lower) of PGE₂ (left). Temperature threshold for TRPV1 activation (broken lines) in the presence of PGE₂ (30.6 ± 1.1 °C) was significantly lower than that in the absence of PGE₂ (40.7 ± 0.3 °C) (right). * p < 0.05 versus PGE₂ (–). Modified from Moriyama *et al.* (2005).

in the sensitization of TRPV1 downstream of PLC activation by such as bradykinin or NGF since amount of PIP₂ is reduced in its hydrolysis to inositol 1,4,5-trisphosphate (IP₃) and diacyl glycerol (DAG) (Fig. 3). It is also known that phospholipase A_2 (PLA₂) is activated downstream of PLC activation, leading to the generation of lipoxygenase (LOX) products, such as 12-HPETE, from arachidonic acid (AA) (Fig. 3). These facts indicate that three different pathways can work to modulate TRPV1 function downstream of PLC activation: a PKC-dependent pathway, a PIP₂-mediated pathway, and a LOX product-mediated pathway. It is not clear which pathway is predominantly functioning *in vivo*.

C. Sensitization by Other Mechanisms

High-proton concentrations (pH < 6) are generated during various forms of tissue injury, including infection, inflammation and ischemia, and such acidification can elicit pain and hyperalgesia. Mild acidification that normally does not open TRPV1 channel directly was found to sensitize TRPV1 and reduce the temperature threshold of TRPV1 activation (Tominaga *et al.*, 1998). CaMKII was also reported to control TRPV1 activity upon phosphorylation of TRPV1

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at S502 and T704 by regulating capsaicin binding (Fig. 4) (Jung *et al.*, 2004). Thus, phosphorylation of TRPV1 by several different kinases seems to control TRPV1 activity through the dynamic balance between the phosphorylation and dephosphorylation. Furthermore, NGF activates the phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated protein kinase (ERK), and then PI3K and ERK have been reported to sensitize TRPV1 (Zhuang *et al.*, 2004). Mechanisms underlying TRPV1 sensitization seems to be very complicated.

V. DESENSITIZATION OF TRPV1

A. Physiological Significance of Desensitization

Capsaicin not only causes pain but also seems to exhibit analgesic properties, particularly when used to treat pain associated with diabetic neuropathies or rheumatoid arthritis (Szallasi and Blumberg, 1999). This paradoxical effect may relate to the ability of capsaicin to desensitize nociceptive terminals to capsaicin as well as to other noxious stimuli following prolonged exposure. At the molecular level, an extracellular Ca²⁺-dependent reduction of TRPV1 responsiveness on continuous vanilloid exposure (electrophysiological desensitization) may partially underlie this phenomenon (Caterina et al., 1997; Szallasi and Blumberg, 1999), although physical damage to the nerve terminal and depletion of SP and CGRP probably contributes to this effect as well. Ca2+- and voltage-dependent desensitization of capsaicin-activated currents has also been observed in rat DRG neurons (Docherty et al., 1996; Liu and Simon, 1996; Koplas et al., 1997; Piper et al., 1999). This inactivation of nociceptive neurons by capsaicin has generated extensive research on the possible therapeutic effectiveness of capsaicin as a clinical analgesic tool (Bernstein, 1987; Maggi, 1991).

Desensitization to capsaicin is a complex process with varying kinetic components: a fast component that appears to depend on Ca^{2+} influx through TRPV1 (Docherty *et al.*, 1996; Liu and Simon 1996; Koplas *et al.*, 1997; Piper *et al.*, 1999) and a slow component that does not. Calcineurin inhibitors reduce TRPV1 desensitization (the slow component), indicating the involvement of Ca^{2+} -dependent phophorylation/dephosphorylation process (Docherty *et al.*, 1996). In agreement with this finding, phosphorylation of TRPV1 by CaM kinase II was reported to prevent its desensitization (Jung *et al.*, 2004). In addition, PKA-dependent phosphorylation of TRPV1 has been reported to mediate the slow component of TRPV1 desensitization (Bhave *et al.*, 2002). TRPV1 becomes dephosphorylated on exposure to capsaicin, and this phosphorylation can be restored by 8bromo-cAMP.

S116 in the amino terminus was found to be a substrate for PKA-dependent phosphorylation (Fig. 4). T370 was also reported to be responsible for PKA-dependent reduction of TRPV1 desensitization (Fig. 4) (Mohapatra and Nau, 2003).

B. Calmodulin-Mediated Desensitization

CaM has also been reported to be involved in Ca²⁺-dependent desensitization of TRPV1. CaM was found to bind to a 35 amino acid segment in the carboxyl terminus of TRPV1 (position at 767-801) (Fig. 4). Disruption of the CaM-binding segment prevented extracellular Ca²⁺-dependent TRPV1 desensitization to brief capsaicin application (Fig. 6), although some desensitization was still observed on more prolonged capsaicin application in cells expressing the mutant (Numazaki et al., 2003). It has also been reported that CaM binds to the first ankyrin-repeat in the amino terminus of TRPV1 (position at 189–222) and to be involved in desensitization (Fig. 4) (Rosenbaum et al., 2004). Whether the amino or carboxyl terminus is more predominantly involved in Ca²⁺-dependent desensitization by CaM is not known. Neither domain contains CaM-binding sites that are obvious from analysis of the primary sequence such as consensus isoleucine-glutamine motif. Ca^{2+} -dependent desensitization is a relatively common feature of many cation channels, including cyclic nucleotide-gated channels (Molday, 1996), L-type Ca²⁺ channels (Peterson et al., 1999; Zuhlke et al., 1999), P/Q type Ca²⁺ channels (Lee et al., 1999), NMDA receptor channels (Ehlers et al., 1996; Zhang et al., 1998), and TRP channels (Chevesich et al., 1997;



FIGURE 6 Extracellular Ca²⁺-dependent desensitization of whole-cell capsaicin-activated currents in HEK293 cells expressing TRPV1. Desensitization observed in the cells expressing wild type (WT) TRPV1 disappears in the cells expressing mutant *TRPV1* lacking 35 amino acids necessary for CaM binding (Δ 35AA). Modified from Numazaki *et al.* (2003).

Scott *et al.*, 1997). It may represent a physiological safety mechanism against a harmful Ca^{2+} overload in the cell, especially during large Ca^{2+} influx through the channels.

It is easy to predict that a change in Ca²⁺ permeability modulates Ca²⁺dependent desensitization. Indeed, a *TRPV1* mutant where Y671 in the sixth TM domain was replaced with K, caused a robust reduction of Ca²⁺ permeability from $P_{\text{Ca/Na}} = 9.0 \pm 1.3$ to 0.8 \pm 0.1, leading to loss of desensitization in the presence of extracellular Ca²⁺ (Fig. 2) (Mohapatra *et al.*, 2003).

C. Modulation by Lipids

Membrane-derived lipids are known to regulate the function of some ion channels, including TRPV1. For example, TRPV1 was shown to be activated by anandamide, OEA, and some of LOX products as described earlier (Zygmunt et al., 1999; Hwang et al., 2000; Ahern, 2003). TRPM7 and G-protein-coupled inwardly rectifying K^+ channels can be inhibited or potentiated, respectively, by the binding of PIP₂ (Huang et al., 1998; Runnels et al., 2002). As described previously, PIP₂ was found to inhibit TRPV1 activity, and such PIP₂-mediated inhibition was released on activation of PLC by metabotropic receptors (Chuang et al., 2001) and the consequent hydrolysis of PIP₂ to DAG and IP₃. A region of TRPV1 (amino acids 777– 820) having eight positively charged residues was identified as a motif involved in regulation by PIP₂ (Fig. 4) (Prescott and Julius, 2003) possibly because it interacts with the negatively charged head group of the phospholipid. This region includes S800, one of the substrates for PKC-dependent phosphorylation and overlaps with the 35 amino-acid segment necessary for CaM binding. This region might thus be very important for regulation of TRPV1 function.

VI. CONCLUSIONS

Extensive electrophysiological and mutational analyses revealed the gating and regulation mechanisms of TRPV1, and many regions and amino acids involved in TRPV1 function have been identified. Given that fact TRPV1 is a key molecule in peripheral nociception, regulation of TRPV1 function will lead to the development of novel antinociceptive or anti-inflammatory agents. Crystallographic analysis promises to provide even more information about the structural determinants of TRPV1 function.

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CHAPTER 7

TRP Channels as Thermosensors

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I. OVERVIEW

Advances in channel studies have revealed that the sensations of temperature (hot, warm, cool, or cold) are transduced by distinct transient receptor potential (TRP) channels that are expressed by sensory neurons. The best example among these studies is TRPV1, which responds to high temperatures. Following the identification of TRPV1, other TRP channels were found to encode sensitivity to hot, warm, cool, and cold temperatures. Like TRPV1, TRPV2 is also activated by high temperatures over 52°C. TRPV3 shows a high degree of homology to TRPV1 and is activated by warm temperatures with a threshold of 33°C. TRPV3 is expressed in sensory neurons and keratinocytes located in close proximity to free nerve endings, indicating that the signaling of warm stimuli may involve both direct and indirect activation of sensory nerves. TRPV4 originally known to be activated by hypotonic shock but now is known to be activated by warm temperature ranging from 24°C to 34°C. TRPM8 present in a subpopulation of small to medium diameter sensory neurons is activated by reducing the temperature to $<23^{\circ}$ C. Thermal activation of TRPM8 is modulated by cooling compounds, menthol and icilin. TRPA1 (or ANKTM1) is another TRP channel that is activated by cooling at $\sim 16^{\circ}$ C and by icilin and some pungent compounds. TRPA1 is present in a subpopulation of TRPV1expressing sensory neurons. Thus, the activation temperature range for TRP channels spans from noxious cold through cool and warm to noxious heat, illustrating the importance of this ion channel family in sensory transduction. In this chapter, TRP channels that are involved in temperature sensing are introduced along with their biophysical properties as well as chemical activators.

II. INTRODUCTION

Mammals display graded neuronal activity and behavioral responses to a wide range of temperatures. In this way we detect and discriminate between temperatures evoking the sensations of noxious cold, cool, warm, and noxious heat. This temperature sensitivity is encoded in the periphery as recordings from single nerve fibers have identified neurons that fire specifically in response to different temperature stimuli. Noxious heat (>40°C) activates two types of responses in mammalian primary afferent neurons that are characterized by different threshold temperatures. One response has a threshold of about 42–45°C and the other about 49–51°C (Treede *et al.*, 1995). Similarly, some nerve fibers in intact preparation respond to warming (LaMotte and Campbell, 1978; Hallin *et al.*, 1982; Darian-Smith, 1984),

while others are activated by temperatures that we class psychophysically as cool (Dubner *et al.*, 1975; Darian-Smith, 1984; Campero *et al.*, 2001). A final group respond when temperatures are cooled to levels that evoke the sensation of noxious cold (Campero *et al.*, 1996). Such observations point to the existence of distinct transduction mechanisms responsible for thermosensation across the thermal spectrum.

All ion channels are sensitive to changes in temperature and there have been several suggestions that the temperature sensitivity of afferent nerves is encoded by voltage-gated ion channels (Adair, 1999; Viana *et al.*, 2002). A role for such channels is not ruled out, and it is clear that the overall firing of nerves will be influenced by their temperature sensitivities. However, the weight of evidence indicates that the majority of thermal responses are transduced by specialized channels that are members of the TRP channel superfamily.

A. General Features of TRP Channels

TRP channels constitute a large family of ion channels with a wide range of confirmed and proposed physiological functions. In humans, 28 different TRP genes have been identified and these can be grouped into 6 families. The classical TRP channels (TRPCs), the vanilloid receptor related TRP channels (TRPVs), the melastatin or long TRPs (TRPMs), the mucolipins (TRPMLs), the polycystins (TRPPs), and ankyrin transmembrane protein related channels (TRPA). They share the common structural feature of six predicted transmembrane domains with a putative pore loop between transmembrane domains 5 and 6 and a variable number ankyrin repeats in the cytoplasmic N-terminal region (none for TRPMs, 3-4 for TRPCs, 3-5 for TRPVs, 8-18 for TRPAs). These ankyrin-rich regions probably serve a role in protein-protein interactions. Similarly the cytoplasmic C-terminal region has been implicated in other protein interactions and in processes that regulate channel activity. The functional channels are generally considered to be tetramers—in some cases homomeric channel complexes and in others heteromeric channels usually formed by 2 (or 3) members of the same family (e.g., TRPC4/5). These are thought to interact with other proteins at the membrane to form functional supramolecular complexes that control the expression, stability, and signaling activity of the ion channels.

TRP channels play a role in a number of sensory systems: vision, taste, smell, hearing, mechanosensation, and thermosensation and in some cases appear to be polymodal receptors that can transduce more than one type of stimulus. This review focuses on the properties of the thermosensitive channels and their likely roles in nociception induced by different stimuli.

III. TRPV1 IS A NOXIOUS HEAT SENSOR

A major characteristic of polymodal nociceptive neurons is their ability to respond to noxious heat and mechanical stimuli as well as the pungent chili pepper ingredient, capsaicin. The pioneering investigations of noxious heat responses in isolated dorsal root ganglion (DRG) neurons (Cesare and McNaughton, 1996) and the cloning and characterization of TRPV1, previously termed vanilloid receptor 1 (VR1) (Caterina *et al.*, 1997; Tominaga *et al.*, 1998), demonstrated that this channel is the receptor/transducer molecule for capsaicin and noxious heat. Such studies revolutionized our understanding of thermosensitivity and stimulated the search for other thermosensitive channels.

A. Heat Responses in DRG Neurons

Two types of thermally evoked currents have been identified in DRG neurons with different temperature thresholds (Nagy and Rang, 1999a; Ahluwalia *et al.*, 2002). One subpopulation of small diameter DRG neurons responds to heating above a threshold temperature of about 42–43°C with a depolarizing inward current (Cesare and McNaughton, 1996; Vyklicky *et al.*, 1999; Nagy and Rang, 1999a,b; Vlachova *et al.*, 2001, 2003). This current shows an unusually high-temperature sensitivity with a Q₁₀ value of about 18–25 (Fig. 1A), unlike the capsaicin or BK-evoked currents recorded in DRG neurons, which have a Q₁₀ of 1.6–3.9 over a wide range of temperatures extending into the noxious heat range (Cesare and McNaughton, 1996; Vlachova *et al.*, 2001). The other population has a higher threshold and probably corresponds to DRG neuron expressing TRPV2 as discussed later in this chapter.

In general, the finding of a strong positive correlation between noxious heat sensitivity and responsiveness to capsaicin at the whole cell level (Kirschstein *et al.*, 1997; Nagy and Rang, 1999a) points toward a role for the capsaicin receptor in thermosensation. This was demonstrated directly with the cloning of rat TRPV1 when it was shown that heterologous expression of TRPV1 in either *Xenopus* oocytes or mammalian cells conferred sensitivity to capsaicin, low pH, and noxious heat (Fig. 1B) (Caterina *et al.*, 1997; Tominaga *et al.*, 1998). Like native sensory neurons, TRPV1 expressing cells show a temperature threshold of about 42°C and a Q₁₀ value of ~20–27 at suprathreshold temperatures (Tominaga *et al.*, 1998; Welch *et al.*, 2000; Liu *et al.*, 2003). Other properties of the TRPV1 channels and heat sensitive DRG channels are also essentially identical, although a detailed comparison is not possible as most data on TRPV1 has been obtained



FIGURE 1 (A) Current response of rat DRG neuron to a heating ramp from 37°C. (Reprinted from Nagy and Rang, 1999a.) (B) TRPV1 transfected oocytes respond to capsaicin and noxious temperatures. (Reprinted from Tominaga *et al.*, 1998.) (C) Bradykinin augments the heat-evoked current in a rat DRG neuron. (Reprinted from Cesare and McNaughton, 1996.) (D) Bradykinin lowers the thermal threshold for activation of heterologously expressed TRPV1. (Reprinted from Sugiura *et al.*, 2002.)

with capsaicin rather than heat as the activator. Nevertheless, direct comparison reveals that the amplitudes and kinetics of heat activated single channel currents in DRG neurons are similar to those of TRPV1 (Nagy and Rang, 1999b; Premkumar *et al.*, 2002; Liu *et al.*, 2003). Similarly, the available information shows that the relative permeabilities of heat activated channels in DRG neurons $P_{\rm Cs}/P_{\rm Na} = \sim 0.9-1.2$; $P_{\rm Ca}/P_{\rm Na} = \sim 1.2-1.3$ (Cesare and McNaughton, 1996; Nagy and Rang, 1999b) are similar to those seen for heterologously expressed TRPV1 (Tominaga *et al.*, 1998).

B. Chemical Activators of TRPV1

Several exogenous and endogenous chemicals activate TRPV1 in addition to capsaicin. Among the exogenous activators are those with a relatively close structural similarity to capsaicin, such as resiniferatoxin and eugenol (Winter *et al.*, 1990; Szallasi *et al.*, 1999; Yang *et al.*, 2003). An important finding is that TRPV1 can be activated by some endogenous, biologically active, arachidonic acid metabolites such as anandamide (Zygmunt *et al.*, 1999; Smart *et al.*, 2000), 12- and 15-(S)-hydroperoxyeicosatetraenoic acids, 5- and 15-(S)-hydroxyeicosatetraenoic acids, and leukotriene B4 (Hwang *et al.*, 2000) as well as amino acid conjugates of arachidonic acid, for example, *N*-arachidonyl-dopamine (Huang *et al.*, 2002; Toth *et al.*, 2003). These findings provide a basis for the physiological involvement of TRPV1 in thermal hyperalgesia in inflammation since production of at least some of these mediators is increased in inflammatory conditions.

C. Structure-Function Studies on TRPV1

The structural elements that confer particular properties on TRPV1 are still the subject of intense investigation. Several sites have been identified as important regions for agonist binding and activation. In one study, Julius and colleagues (Jordt and Julius, 2002) mapped several amino acid residues important for capsaicin activation by exploiting the observation that chicken TRPV1 is responsive to noxious heat, with a normal threshold temperature of ~42°C, but does not respond to capsaicin (Nagy and Rang, 2000; Marin-Burgin et al., 2000). They constructed chimeric channels from rat and chicken TRPV1 to identify regions that conferred this species difference. From this study they were able to conclude that the important residues were in the segment between transmembrane domains 2 and 4. Further site directed mutagenesis experiments showed that a tyrosine residue at position 511 at the proposed interface between the first intracellular loop 2 and transmembrane domain 3 was essential for ³[H]-RTX binding and activation by vanilloid agonists (Jordt and Julius, 2002). Other polar residues, arginine 491 in transmembrane domain 2 and serine 512, had a strong effect on capsaicin activation. These amino acids are putative sites for the binding of vanilloid agonists including the endogenous activator, anandamide.

A similar chimeric receptor/site directed mutagenesis approach using TRPV1 from different species has shown the importance of residues 547 and 550 in transmembrane region 4 for the binding of vanilloid agonists and for some effects of the TRPV1 antagonist, capsazepine (Gavva *et al.*, 2004b). The substitution of a threonine residue at position 550 for isoleucine (I550T) and methionine at 547 for leucine (L547M) confers greater capsaicin sensitivity and higher affinity RTX binding to rabbit TRPV1 than seen for rat TRPV1 (Gavva *et al.*, 2004b). Residue 547 is also important for the agonist actions of the phorbol derivative phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV), which is a strong agonist of rat TRPV1 but not human TRPV1. Mutation of methionine 547 in TM4 of rat TRPV1 to leucine, which is found in human TRPV1 (M547L), reduced the ability of

PPAHV to activate TRPV1 by 20-fold, while the reciprocal mutation of human TRPV1 (L547M) enabled the human receptor to respond to PPAHV (Phillips *et al.*, 2004). Furthermore, residue 547 affects some, but not all, of the inhibitory effects of the TRPV1 antagonist, capsazepine. Capsazepine is a weak antagonist against low pH stimulation at rat TRPV1 but a good antagonist at human TRPV1, and the single residue mutation M547L in rat TRPV1 greatly increased the ability of capsazepine to inhibit low pH evoked responses (Phillips *et al.*, 2004). The ability of capsazepine to inhibit capsaic in responses is relatively unaffected by the mutations, and the data suggest that residue 547 is not important for capsazepine to inhibit channel activation by either low pH or noxious heat.

Despite the importance of the residues at 491, 511, 512, 547, and 550 for the actions of vanilloid-like compounds, substitution of these residues has no major effect on activation by either low pH or heat. There is little data on the structural basis for thermal activation. The cytoplasmic C-terminal segment can certainly influence thermal gating and progressive truncation of the C-terminal (removing 31 or 72 amino acid residues) reduces both the thermal threshold and the Q_{10} of the thermally activated current. (Vlachova *et al.*, 2003). These data suggest a strong interaction between the C-terminal and the gating region to regulate thermal activation. Modifications to the C-terminal region, including phosphorylation as well as experimental truncation can, however, affect activation by chemical agents known to bind, at least in part, to other sites on TRPV1. For this reason it is premature to conclude that the thermal sensor *per se* is located in the intracellular C-terminal region.

D. Biochemical Regulation

The activity of TRPV1 is regulated by several intracellular pathways including phosphorylation of several key residues. TRPV1 phosphorylation leads to increased sensitivity to both chemical and thermal stimuli, which is an important feature since a number of agents produced during inflammation activate protein kinase A (PKA) and/or protein kinase C (PKC). Conversely, dephosphorylation is thought to underlie the calcium-mediated desensitization of both native channels and heterologously expressed TRPV1.

Although TRPV1 contains many putative sites for PKA, PKC, and CaM kinase-mediated phosphorylation, the available biochemical evidence points to key regulatory roles for three major sites leading to increased TRPV1 sensitivity. Serine 502 located in the intracellular loop between transmembrane domains 2 and 3 is phosphorylated by both PKA and

PKC, serine 800 in the C-terminal intracellular tail is a substrate for PKCmediated phosphorylation, and serine 116 in the N-terminal region is phosphorylated by PKA (Bhave *et al.*, 2002; Numazaki *et al.*, 2002; Bhave *et al.*, 2003). Site directed mutagenesis studies indicate that phosphorylation of other residues in the N- and C-terminal regions can contribute to the regulation (Mohapatra and Nau, 2003; Jung *et al.*, 2004), but the biochemical evidence for their involvement in normal or pathophysiological conditions is less well established.

A striking feature of TRPV1 is its calcium mediated inactivation or desensitization, which leads to a loss of sensitivity to capsaicin and other chemical agonists and a reduction in heat sensitivity associated with a reduction in the Q_{10} of the heat-evoked currents (Vyklicky *et al.*, 1999). The biochemical basis for desensitization is probably dephosphorylation of TRPV1 driven by the calcium influx through open TRPV1 channels, which activates the calcium dependent phosphatase, calcineurin (Docherty et al., 1996; Jung et al., 2004; Mohapatra and Nau, 2005). A threonine residue at position 370 in the N-terminal region has been suggested as an important site of dephosphorylation as calcineurin inhibition has no effect on desensitization of a T370A TRPV1 mutant channel (Mohapatra and Nau, 2005), although it is possible that such mutations allosterically affect other PKA/ PKC phopshorylation sites such as those noted above as important for sensitization. Other regions of the channel can also influence desensitization. For example, mutation of a tyrosine residue 671 in transmembrane domain 6 abolishes calcium-mediated desensitization (Mohapatra et al., 2003) for reasons that remain to be explored.

E. TRPV1 in Inflammation

Inflammation is associated with the generation of a host of inflammatory mediators, including bradykinin, prostaglandins, and products of the lipoxygenase enzyme pathway. The extracellular pH is known to be lowered in inflammatory conditions and production of nerve growth factor (NGF) is increased. All these agents, as well as other, less well-cataloged mediators (ATP, proteolytic enzymes) can sensitize TRPV1. The biochemical pathways that underlie sensitization have been the subject of considerable interest.

Low pH can both sensitize and activate TRPV1 by protonation of two glutamate residues in the putative pore domain (E600 and E648, respectively) (Jordt *et al.*, 2000). Other inflammatory mediators are known to stimulate either PKA- or PKC-mediated phosphorylation and in this way can sensitize TRPV1. The sensitization of heat responses was first shown for bradykinin acting on B2 receptors in DRG neurons (Fig. 1C), where the effect could be

mimicked by application of PKC-activating phorbol esters (Cesare and McNaughton, 1996). A similar heat sensitization is seen with heterologously expressed TRPV1 in response to bradykinin and to ATP operating through the G_q coupled P2Y₂ receptor (Fig. 1D) (Tominaga *et al.*, 2001; Numazaki *et al.*, 2002; Sugiura *et al.*, 2002). Subsequent studies have shown that a particular isoform of PKC, PKC-epsilon, mediates and mimics the effects of bradykinin and ATP activation (Cesare *et al.*, 1999; Numazaki *et al.*, 2002).

PKC activation is also thought to underlie the neuronal thermal sensitization evoked by inflammatory proteases, such as mast cell tryptase and trypsins, which activate the phospholipase C (PLC)-linked protease-activated receptor 2 (PAR2) (Amadesi *et al.*, 2004; Dai *et al.*, 2004).

Nerve growth factor can acutely sensitize sensory neurons to heat (Galoyan *et al.*, 2003), as well as exerting a long-term regulation on TRPV1 expression. This acute sensitization, which can also be detected using capsaicin as the stimulus (Bonnington and McNaughton, 2003) can be abrogated by PKC inhibitors (Bonnington and McNaughton, 2003; Galoyan *et al.*, 2003). The pathway leading to sensitization appears more complex than for bradykinin since inhibitors of PLC and of the ras/MEK pathway have no significant inhibitory effect on sensitization, although wortmannin, at concentrations that specifically inhibit phosphatidylinositol-3-kinase (PI3K) totally abolishes the effect of NGF (Bonnington and McNaughton, 2003). These results suggest that TrkA signaling activates a pathway that involves PI3K, while PLC has a role at a subsequent stage in the pathway.

Several inflammatory mediators are throught to sensitize TRPV1 via PKAmediated phosphorylation. These include prostaglandins and glutamate acting indirectly through the metabotropic mGlu5 receptor (Hu *et al.*, 2002).

In addition to phosphorylation, it has been proposed that phosphatidylinositol-4,5-bisphosphate (PIP₂) binds to the C-terminal of TRPV1 in the region of serine 800 and exerts a tonic inhibitory effect on channel function. In this scenario, activation of PLC leads to the cleavage of PIP₂, thereby relieving the inhibition and activating the channel. This mechanism has been proposed as an alternative to the PKC pathway to explain the sensitization/ activation induced by either bradykinin or NGF (Chuang *et al.*, 2001; Prescott and Julius, 2003). PLA₂ mediated PIP₂ hydrolysis may also lead to the production of lipoxygenase derived lipid mediators, which can directly gate TRPV1 (see an earlier section).

F. Lessons from "Knockout" Mice

The behavioral effects of TRPV1 deletion have been demonstrated in two major independent studies (Caterina *et al.*, 2000; Davis *et al.*, 2000).

Both studies demonstrated a large reduction in thermal, but not mechanical, hyperalgesia in TRPV1-null mice during inflammation. In contrast, wild type and TRPV1-null mice developed similar levels of thermal or mechanical hyperalgesia after nerve injury indicating that different mechanisms underlie heat sensitization in inflammatory and neuropathic conditions. The acute response to high temperatures is reduced in mice lacking TRPV1 but only at very high temperatures (>48 $^{\circ}$ C), which is surprising given that the noxious heat threshold of TRPV1 is about 42°C and that isolated DRG neurons from TRPV1-null mice do not respond until the temperature exceeds 50°C (Caterina et al., 2000). The more intact skin-nerve preparation does, however, retain a population of nerve fibers that respond with a normal noxious temperature threshold, albeit firing at a lower rate. This finding led to the suggestion that other thermosensitive TRP channels are important for encoding noxious heat at the lower noxious temperatures in intact tissue systems. TRPV2 was considered a good candidate for this high-threshold response (see a later section), but this now seems unlikely given that nociceptors lacking both TRPV1 and TRPV2 have normal noxious heat responses (Woodbury et al., 2004). This leaves other mechanisms, perhaps TRPV3 or TRPV4, to encode the responses to low noxious heat stimuli.

G. Effects of TRPV1 Antagonists

Because TRPV1 is thought to play an important role in nociception, a number of groups have sought to discover selective TRPV1 antagonists. Several chemically diverse classes of antagonist have now been identified and tested for effects *in vivo*. These data show a good degree of consistency indicating that the effects are mediated via TRPV1 inhibition.

TRPV1 antagonists have little effect on acute thermal nociception. They do, however, reduce the thermal hyperalgesia associated with complete Freund's adjuvant (CFA)-induced inflammation (Pomonis *et al.*, 2003; Walker *et al.*, 2003; Gavva *et al.*, 2004a; Gomtsyan *et al.*, 2005) and decrease the number of abdominal constrictions induced by intraperitoneal administration of either acetic acid (Gomtsyan *et al.*, 2005) or phenyl-*p*-quinone (Suh *et al.*, 2003).

One surprising and potentially important finding is that TRPV1 antagonists reduce mechanical hyperalgesia induced by CFA (Pomonis *et al.*, 2003; Walker *et al.*, 2003; Gavva *et al.*, 2004a). Capsazepine, as well as BCTC, also reduce mechanical hyperalgesia and tactile allodynia 2 weeks after partial sciatic nerve injury (Pomonis *et al.*, 2003; Walker *et al.*, 2003). The link to mechanical hypersensitivity is unclear and may not reside at the level of TRPV1 or the peripheral neurons but rather may reflect a sensitization process in the spinal cord.

H. Regulation of TRPV1 Expression

The expression levels of TRPV1 are regulated by environmental factors, notably the levels of the neurotrophins-NGF, brain derived neurotrophic factor (BDNF), and glial derived neurotrophic factor (GDNF)-as well as neuronal damage. NGF controls TRPV1 expression by acting via the highaffinity (TrkA) NGF receptor expressed by a subset of sensory nerves. Withdrawal of NGF either in culture (Bevan and Winter, 1995) or by the use of neutralizing TrkA-IgG fusion molecule in vivo (McMahon et al., 1995) leads to a loss of response to capsaicin. Conversely, provision of high levels of NGF in vitro or by over-expression in vivo leads to elevated TRPV1 responses, probably by increasing the level of channel expression. The production of NGF by peripheral, non-neuronal tissues is increased during inflammation (Otten, 1991) and leads to increased TRPV1-mediated capsaicin sensitivity (Nicholas et al., 1999). TRPV1 expression is also regulated by other neurotrophins (BDNF and GDNF) in sensory neurons that express the appropriate receptors (Winter, 1998; Ogun-Muyiwa et al., 1999). Increased neurotrophin supply is thought to underlie the upregulation of TRPV1 immunoreactivity in experimental inflammation (Ji et al., 2002; Amaya et al., 2004) and may be responsible for the upregulation of TRPV1 expression in human inflammatory diseases (Yiangou et al., 2001). This long-term transcriptional control of TRPV1 expression augments the acute sensitizing actions of the various inflammatory mediators, including NGF noted above.

TRPV1 expression is also controlled by nerve damage and this may also be linked to the local supply of neurotrophins. Axotomy, which will deprive the neurons of their peripheral neurotrophin supply, leads to a loss of capsaicin sensitivity and TRPV1 expression (Michael and Priestley, 1999). Partial nerve damage has a more subtle and interesting effect. As expected, TRPV1 expression is downregulated in the damaged nerve fibers, but expression levels are upregulated in the adjacent undamaged neurons. This increase in expression is not restricted to C-fibers but expression has been noted in a significant number of undamaged A-fibers (Hudson *et al.*, 2001; Rashid *et al.*, 2003). This changed pattern of expression may underlie the abnormal thermal sensations seen after nerve injury.

I. TRPV1 Expression in Other Neuronal and Non-Neuronal Cells

Although TRPV1 was thought to be uniquely expressed in nociceptive afferent neurons, there is now abundant evidence for expression in some other regions in the brain as well as in some non-neuronal cells throughout the body. TRPV1 is expressed in the hypothalamus, which is an important region of the brain for temperature regulation. Injection of capsaicin directly into the hypothalamic region evokes a hypothermia (Jancso-Gabor *et al.*, 1970a,b) that is lost in TRPV1 null mice (Caterina *et al.*, 2000). TRPV1 mRNA and protein have been detected in this brain region (Sasamura and Kuraishi, 1999; Mezey *et al.*, 2000). It is unclear how TRPV1 contributes to temperature regulation given that the threshold for activation is far higher than normal body temperature and mice lacking TRPV1 show normal body temperatures (Caterina *et al.*, 2000). One possibility is that some endogenous mediator lowers the thermal threshold of TRPV1 in the hypothalamus so that the channel is operational at normal physiological temperatures (Caterina, 2003). However, the available data strongly suggest that other non-TRPV1 mechanisms must play an important role in thermoregulation.

TRPV1 mRNA, protein or binding sites for the high-affinity TRPV1 ligand, resiniferatoxin, have been detected in several other brain regions such as the locus coereleus, olfactory nuclei, some regions of the cerebral cortex, dentate gyrus, central amygdala, habenula, striatum, centromedial, and paraventricular thalamic nuclei, substantia nigra, reticular formation, periaqueductal grey, superior colliculus, and inferior olive (Roberts *et al.*, 2004). Whether TRPV1 in these sites has a functional role is unclear.

TRPV1 expression has also been demonstrated in parts of the the inner ear, including inner and outer hair cells, inner and outer pillar cells, Hensen's cells and satellite cells (Balaban *et al.*, 2003; Zheng *et al.*, 2003). In addition, human cultured keratinocytes (Inoue *et al.*, 2002; Southall *et al.*, 2003) as well as epithelial cells in the stomach (Kato *et al.*, 2003) and urinary bladder (Birder *et al.*, 2001) have been reported to express functional TRPV1 channels. Once again the physiological roles of TRPV1 in these locations is obscure.

IV. TRPV2

TRPV2 originally known as VRL-1 was the second thermosensitive TRP channel to be discovered and characterized (Caterina *et al.*, 1999). This 761–764 amino acid protein shows approximately 50–66% amino acid identity with rat and human TRPV1, respectively. Like TRPV1, it is also activated by high temperatures, although in this case the thermal threshold is about 52°C (Fig. 2B). This high threshold is similar to the higher thermal threshold seen in a subpopulation of rat isolated DRG neurons (Fig. 2A) (Nagy and Rang, 1999a; Ahluwalia *et al.*, 2002) and in mechanoheat sensitive A-fibers in monkey skin (Treede *et al.*, 1995). TRPV2 is not activated by low pH, or by capsaicin, or other typical TRPV1 agonists (Fig. 2A) but, like TRPV1



FIGURE 2 (A) Currents evoked in capsaicin sensitive (left) and capsaicin-insensitive (right) DRG neurons by heat ramps from 37° C, showing the higher thermal threshold of the capsaicin-insensitive neuron. Bottom traces show responses to 2 μ M capsaicin. (Reprinted from Nagy and Rang, 1999a.) (B) Heat evoked current responses of TRPV2 (VRL-1) and TRPV1 (VR1) transfected HEK293 cells showing the higher threshold of TRPV2. (Reprinted from Caterina *et al.*, 1999.)

and TRPV3 (see a later section), can be activated by 2-aminoethoxydipheny borate (2-APB) (Hu *et al.*, 2004). This activation by an exogenous chemical activator raises the possibility that TRPV2 channel opening can be stimulated or modulated by endogenous mediators.

TRPV2 channels are nonselective monovalent and divalent cation permeable channels ($P_{\rm K}/P_{\rm Na} = 0.86$, $P_{\rm Cs}/P_{\rm Na} = 0.95$, $P_{\rm Mg}/P_{\rm Na} = 2.4$, $P_{\rm Ca}/P_{\rm Na} =$ 2.9), show a typical outward rectification, and can be blocked by ruthenium red (EC₅₀ 0.6 μ M) but not by capsazepine (Caterina *et al.*, 1999), thereby differentiating them from TRPV1.

A. Expression Pattern

Immunocytochemical studies have shown that TRPV2 is expressed mainly in medium to large diameter neurons in rat DRG (Caterina *et al.*, 1999; Ma, 2001; Ahluwalia *et al.*, 2002; Lewinter *et al.*, 2004) with little co-staining for substance P or the isolectin B4 marker for small diameter nociceptive neurons. In general, there is little overlap between TRPV1- and TRPV2expressing cells (Ahluwalia *et al.*, 2002), although one paper has reported that 20% of TRPV2 positive DRG neurons also express TRPV1 (Greffrath et al., 2003). About a third of TRPV2 positive rat DRG neurons co-stain with an antibody directed against CGRP, which suggests staining of unmyelinated C-fibers, while many TRPV2 positive neurons can be labeled by the neurofilament antibody N52, which is a marker of myelinated DRG neurons. Similarly a high percentage of TRPV2-expressing rat trigeminal neurons that innervate the tooth pulp (45%) or facial skin (25%) express CGRP (Ichikawa and Sugimoto, 2000). In adult mice about 70% of TRPV2 expressing DRG neurons also express the neurotrophin receptor, TrkC, while 25% express the GDNF receptor, Ret (Tamura et al., 2005). Whether TRPV2 expression is regulated by neurotrophins is currently unknown, although this seems unlikely, given the observation that axotomy in the spinal nerve ligation model does not lead to a reduction in TRPV2 in either the damaged or adjacent undamaged DRG neurons (Gaudet et al., 2004). In this way TRPV2 expression appears independent of a neurotrophin supply, unlike the regulation seen for TRPV1.

B. Other Activators of TRPV2

A non-thermosensitive role for TRPV2 is suggested by its detection by immunostaining in a range of cell types including enteric neurons in the intestine (Kashiba *et al.*, 2004), sympathetic neurons (Gaudet *et al.*, 2004), motoneurons and ventral root efferent neurons (Lewinter *et al.*, 2004), aortic smooth muscle cells (Muraki *et al.*, 2003) as well as mRNA expression in lung, spleen, intestine, brain (Caterina *et al.*, 1999), a mast cell line (Stokes *et al.*, 2004), and hepatocytoma cells (Vriens *et al.*, 2004a). The physiological roles of TRPV2 in these and other tissues remain to be explored, but it is noteworthy that the mouse ortholog of TRPV2 (growth-regulated channel GRC) translocates from the cytosol to the plasma membrane after stimulation with insulin-like growth factor (Kanzaki *et al.*, 1999) and can be activated by membrane stretch and hypotonic stimulation (Iwata *et al.*, 2003; Muraki *et al.*, 2003). The latter observation clearly suggests some putative role in mechanotransduction that requires further investigation.

V. TRPV3 AND TRPV4 ACT AS WARM RECEPTORS

Both TRPV3 and TRPV4 show thermosensitive properties and cellular expression patterns that are consistent with roles as warm thermosensors. TRPV4 was described before TRPV3 and shows a wider cellular distribution in different tissues and greater complexity of gating mechanisms, indicating

additional non-thermosensitive roles. For these reasons TRPV4 has been studied more extensively than TRPV3.

Although it would seem likely that warm sensations are sensed directly by a subset of primary afferent neurons, there is little convincing evidence that isolated DRG neurons respond to warm stimuli (Gotoh *et al.*, 1998). It has therefore been suggested that warm sensations are sensed by non-neuronal cells, especially as both TRPV3 and TRPV4 channels are expressed abundantly in skin cells. In this scenario, non-neuronal cells respond to a warm stimulus by releasing mediators that signal to the free nerve endings of skin afferents, which lie in close proximity to the skin cells (Peier *et al.*, 2002b). Keratinocytes can release ATP that excites afferent neurons (Koizumi *et al.*, 2004) and mice lacking the P2X3 receptor normally expressed in sensory afferents fail to respond to warm stimuli (Souslova *et al.*, 2000). These findings suggest keratinocyte-derived ATP as a candidate mediator for this signaling pathway. Further experiments are necessary to determine whether or not this hypothesis has any validity.

A. TRPV3

TRPV3 is a ~790 amino acid protein with about 43% amino acid identity to TRPV1 and TRPV4 and 41% to TRPV2 (Smith *et al.*, 2002; Peier *et al.*, 2002b). Like the other thermosensitive TRP channels, TRPV3 is a nonselective cation channel: $P_{\rm Cs}/P_{\rm Na} \cong P_{\rm K}/P_{\rm Na} \cong 1$ with $P_{\rm Ca}/P_{\rm Na} = 2.6-12$ and $P_{\rm Mg}/P_{\rm Na} = 2.2$ (Xu *et al.*, 2002; Peier *et al.*, 2002b). The single channel conductance is large with estimates ranging from 170 pS (Xu *et al.*, 2002) to 337 pS (Chung *et al.*, 2004a).

1. Thermal and Chemical Activation

TRPV3 is not responsive to either hypotonicity or the phorbol ester derivative, 4α -phorbol 12,13-didecanoate (4α -PDD) that activates TRPV4 (Smith *et al.*, 2002; Xu *et al.*, 2002; Watanabe *et al.*, 2002a; Peier *et al.*, 2002b). TRPV3 is not activated by low pH, capsaicin and resiniferatoxin that activate TRPV1. However, heterologously expressed TRPV3 channels are activated by warming with estimated threshold temperatures ranging from ~31–33°C (Xu *et al.*, 2002; Peier *et al.*, 2002b) to 39°C (Smith *et al.*, 2002). The evoked membrane current continues to increase in amplitude as the temperature is increased into the noxious range (Fig. 3A) with a high Q₁₀ of 7–17 (Xu *et al.*, 2002; Peier *et al.*, 2002b). The continued increase in current with noxious temperatures differs from that seen with TRPV4, which desensitizes as the temperature reaches the noxious range. This result suggests that TRPV3 can possibly code for noxious as well as non-noxious heat.



FIGURE 3 (A) Current evoked in TRPV3 expressing CHO cell by heating with a threshold temperature of about 33°C. Note no inactivation of the current at high temperatures. (B) Repeated heat stimuli lead to sensitization of TRPV3 mediated currents. (Reprinted from Peier *et al.*, 2002b.)

Temperature activated TRPV3 currents show a pronounced outward rectification in physiological medium with relatively little inward current (Smith *et al.*, 2002; Peier *et al.*, 2002b). In part this is due to calcium block of the channel at negative membrane potentials as much larger inward currents are seen when calcium is removed from the extracellular solution. This calcium sensitivity may have physiological significance as the extracellular calcium concentration in parts of the epidermis is low (Elias *et al.*, 2002).

Relatively little is known about the intracellular mechanisms controlling TRPV3 activity, but responses to heat stimulation increase with repeated heat application indicating a sensitization process (Fig. 3B) (Peier *et al.*, 2002b).

The pharmacological properties of TRPV3 have been largely unexplored. Responses are blocked by ruthenium red but not capsazepine (Smith *et al.*, 2002; Xu *et al.*, 2002; Peier *et al.*, 2002b). Camphor (Moqrich *et al.*, 2005) and 2-APB (Hu *et al.*, 2004; Chung *et al.*, 2004a), which is a blocker of store-operated calcium channels, are the only known chemical activators. The responses to these agonists sensitize with repeated administration and also cross sensitize to heat. The effect of 2-APB also shows synergy with heat (Hu *et al.*, 2004; Chung *et al.*, 2004a). In primary keratinocytes, 2-APB significantly increases the percentage of primary keratinocytes exhibiting TRPV3-like heat evoked currents, and this augmentation is retained in keratinocytes from TRPV4 null mice (Chung *et al.*, 2004a). The rapid action of external 2-APB and the lack of any agonism or desensitization to heat activation

when 2-APB is applied internally through the recording pipette in electrophysiology experiments argues that the 2-APB binding site is external (Hu *et al.*, 2004).

2. Distribution of TRPV3

A low level of TRPV3 expression has been reported for sensory neurons. In fact Peier *et al.* (2002b) reported no significant expression levels in mouse DRG neurons, although TRPV3 can be detected by PCR in these tissues. TRPV3 expression was detected by immunocytochemistry in human DRG neurons, albeit in a lower percentage (20%) of smaller diameter (<50 μ M) neurons than TRPV1 (~70%) (Smith *et al.*, 2002), and *in situ* hybridization revealed TRPV3 expression in almost all monkey DRG and trigeminal ganglion neurons (Xu *et al.*, 2002). One study showed that the level of TRPV3 was increased twofold in human DRG after brachial plexus avulsion (Smith *et al.*, 2002).

TRPV3 is expressed abundantly in skin, notably in keratinocytes in the epidermis and in hair follicle cells and has also been detected by both *in situ* hybridization and immunocytochemistry in primary keratinocyte cultures and in keratinocyte cell lines (Xu *et al.*, 2002; Peier *et al.*, 2002b; Chung *et al.*, 2003, 2004b). A high level of expression has been reported in the CNS (Smith *et al.*, 2002) and *in situ* hybridization studies have also shown expression in ventral motoneurons and in deeper, but not superficial, laminae of the spinal cord as well as sympathetic neurons of the superior cervical ganglion (Xu *et al.*, 2002). The physiological significance of expression in these locations is unclear but such findings suggest additional non-thermosensitive roles for TRPV3.

B. TRPV4

Mammalian TRPV4 is an 871–873 amino channel with the typical TRPV family structure (Liedtke *et al.*, 2000; Strotmann *et al.*, 2000). The channel does not differentiate significantly between small monovalent cations ($P_{\rm K}/P_{\rm Na} = 1.2-1.4$; $P_{\rm Cs}/P_{\rm Na} = 1.1-1.3$; $P_{\rm Li}/P_{\rm Na} = 0.85$) and shows a higher permeability to calcium ($P_{\rm Ca}/P_{\rm Na} = 5.8-9.3$) and magnesium ions ($P_{\rm Mg}/P_{\rm Na} = 2.5$) (Voets *et al.*, 2002; Watanabe *et al.*, 2002a; Strotmann *et al.*, 2003; Suzuki *et al.*, 2003a). The calcium permeability of the channel is due, in part, to the presence of two aspartate residues at positions 672 and 682 in the putative pore domain. These residues are in a stretch of 13 amino acids, which are highly conserved across TRPV1, TRPV2, and TRPV4. This region of the pore is

also responsible for the voltage dependent channel block by ruthenium red (Strotmann *et al.*, 2000; Voets *et al.*, 2002; Watanabe *et al.*, 2002a).

The current–voltage relationship of TRPV4 shows both inward and outward rectification in normal physiological solutions. This rectification is dependent on the presence of external Ca (Liedtke *et al.*, 2000; Voets *et al.*, 2002) and is partly due to the presence of the two aspartate residues at positions 672 and 682 noted above as the strong Ca-dependent rectification is much reduced when both these residues are mutated to alanine (Voets *et al.*, 2002). The reported single channel conductance varies from 310 pS at +80 mV seen in both inside-out and outside-out excised patches (Liedtke *et al.*, 2000) to 30 pS at -60 mV and 88 pS at +60 mV in cell attached patches (Strotmann *et al.*, 2000).

1. Expression of TRPV4

The expression pattern of TRPV4 is consistent with its proposed role as an osmo- or volume-sensor. TRPV4 has been identified in epithelial cells in a wide range of tissues including kidney tubules (Liedtke *et al.*, 2000; Strotmann *et al.*, 2000; Delany *et al.*, 2001), bronchii, and lung (Delany *et al.*, 2001; Jia *et al.*, 2004; Tian *et al.*, 2004), sweat glands (Delany *et al.*, 2001), in cochlea hair cells and osmosensory cells of the circumventricular organs of the brain (Liedtke *et al.*, 2000) as well as in the pre-optic/anterior hypothalamus (Guler *et al.*, 2002) and heart and vascular endothelial cells. TRPV4 is also expressed in keratinocytes (Guler *et al.*, 2002; Suzuki *et al.*, 2003b), which is significant in respect to its potential role as a thermosensor.

In contrast to the clear expression in non-neuronal cells, reports of expression in sensory neurons have been less consistent and the data suggest a relatively low neuronal expression level. mRNA for TRPV4 has been detected in rat DRG and trigeminal ganglia (Guler et al., 2002) but not in human DRG (Delany et al., 2001). No specific immunostaining was seen in rat or human DRG cell bodies or nerve fibers (Delany et al., 2001; Guler et al., 2002), although protein expression has been noted in peripheral nerves by Western blotting after nerve ligation, which concentrates the proteins by inhibiting transport from the soma to the periphery (Alessandri-Haber et al., 2003). In another study, TRPV4 immunostaining was also observed in about 10% of L4/5 DRG neurons, mainly coexpressed with NF200, a marker for large and medium sized A-fiber sensory neurons (Suzuki et al., 2003b). No staining was noted in sensory nerve terminals in the spinal cord suggesting that TRPV4 is preferentially transported toward the periphery. TRPV4 staining has been noted in Merkel cells and Meissner corpuscles and in the sensory fibers innervating these structures as well as other sensory nerve endings with a mechanosensitive role (Liedtke et al., 2000; Suzuki et al., 2003b).

Positive immunostaining has also been noted in human sympathetic and parasympathetic nerve fibers innervating the skin, sweat glands, intestine, and blood vessels (Delany *et al.*, 2001).

2. Functions of TRPV4: Chemical and Osmotic Activation

TRPV4 was originally identified as a channel that was activated by cell swelling induced by exposure to hypotonic medium in both heterologous expression systems and native cells (Liedtke et al., 2000; Strotmann et al., 2000; Nilius et al., 2001). Subsequent investigations revealed that TRPV4 can be activated by a range of stimuli including the phorbol ester derivatives, 4α -PDD and phorbol 12-myristate 13-acetate (PMA) at low micromolar or submicromolar concentrations (Watanabe et al., 2002a; Gao et al., 2003) and low pH (pH < 6) (Suzuki *et al.*, 2003a). The effect of the phorbol esters appears to involve two pathways. One pathway is temperature independent and is not linked to PKC activation as 4a-PDD, unlike PMA, does not activate PKC. The other pathway is apparent at higher, more physiological temperatures and is PKC-mediated as it is blocked by the PKC inhibitors, bisindolylmaleimide, staurosporine, and calphostin C (Gao et al., 2003; Xu et al., 2003). At present there is no evidence for the involvement of the PKC pathway in the hypotonic response of TRPV4 since this is insensitive to PKC inhibitors and to downregulation of PKC by prolonged exposure to phorbol esters (Gao et al., 2003; Xu et al., 2003).

TRPV4 is also activated by arachidonic acid, anandamide, and 2-arachidonyl glycerol (2-AG). Unlike 4α -PDD, the effects of these lipid mediators are not membrane delimited as they do not activate TRPV4 channels in inside-out membrane patches (Watanabe *et al.*, 2003b). The effect of anandamide (and 2-AG) is dependent on hydrolysis by the enzyme fatty acid amide hydrolase (FAAH) to generate arachidonic acid. Activation of TRPV4 by arachidonic acid is not attenuated by lipoxygenase or cyclooxygenase inhibitors but is inhibited by blockers of cytochrome P450 epoxygenase (e.g., miconazole and 17-octadecanoic acid). The epoxyeicosatrienoic acids (EETs), 5',6'-EET and to a lesser extent 8',9'-EET, generated from arachidonic acid by the epoxygenase pathway activate TRPV4 in both heterologous expression systems and native cells (Vriens *et al.*, 2004b). The finding that 5',6'-EET activates single channel currents with a typical TRPV4 conductance (50–60 pS) in inside-out membrane patches suggests that this agent acts directly on the channel complex (Vriens *et al.*, 2004b).

Cell swelling is known to activate PLA₂ (Thoroed *et al.*, 1997; Pedersen *et al.*, 2000) and PLA₂ inhibition greatly reduces or abolishes the hypotonicityinduced Ca²⁺ response and whole cell currents in TRPV4 expressing cells, but has no effect on the amplitude of the responses evoked by heat, 4α -PDD, arachidonic acid, or 5',6'-EET (Vriens *et al.*, 2004b). The response to hypotonicity is also blocked by the cytochrome P450 epoxygenase inhibitors, miconazole and 17-ODYA, consistent with a scheme where cell swelling activates PLA_2 to generate arachidonic acid which is then metabolized by cytochrome P450 epoxygenase to an EET, such as 5',6'-EET, which activates TRPV4 (Vriens *et al.*, 2004b).

In contrast to the effects of arachidonic acid and hypotonicity, activation of TRPV4 by heat or 4α -PDD is insensitive to miconazole and 17-ODYA, indicating a different activation mechanism for these stimuli (Vriens *et al.*, 2004b).

3. Heat Responses of TRPV4

Although some studies have failed to show that heterologously expressed TRPV4 is thermosensitive under normal conditions (Liedtke *et al.*, 2000; Strotmann *et al.*, 2000; Suzuki *et al.*, 2003a), an interaction between osmotic activation and temperature was noted in an early study (Liedtke *et al.*, 2000). Investigations have revealed that TRPV4 is activated by temperatures in the warm range.

Raising the temperature of the solution bathing TRPV4 expressing *Xenopus* oocytes evokes a cationic current with a threshold temperature of $\sim 27^{\circ}C$ (Fig. 3A) and always at temperature lower than those required to activate TRPV1 (Guler et al., 2002). In a similar manner, HEK293 cells either transiently or stably expressing TRPV4 respond to heating with a Ca response and inward currents that are inhibited by ruthenium red (Guler et al., 2002; Watanabe et al., 2002b). Once again the threshold temperatures are lower than those for TRPV1, with thresholds as low as 24–34°C, and the current amplitude continues to increase as the temperature is raised, although desensitization often obscures the true temperature-response relationship. The TRPV4 heat-evoked current shows a high Q₁₀ of 9.9-19.1 (Guler et al., 2002; Watanabe et al., 2002b). Repeated heat stimulation leads to desensitization of the whole cell responses with a shift in threshold to higher temperatures, from 24°C to 30°C. In addition, heat activation is lost in mutants where the 2 ankyrin repeat domains are deleted (Watanabe et al., 2002b), which suggests that the ankyrin repeats are necessary to stabilize a channel conformation that is permissive for heat activation.

The single channel conductance of heat activated TRPV4 (61 pS at -60 mV and 99 pS at +60 mV in cell attached patches) is similar to the conductance of 4α -PDD-activated channels (59 pS at -60 mV). However, no heat activated single channels were seen in cell free inside-out patches although 4α -PDD is able to activate single channel activity in this experimental configuration (Watanabe *et al.*, 2002b). These results suggest that heat activates TRPV4 via the production of a chemical ligand rather than by a direct gating of the channel.

In the HEK293 cell expression system, the thermal response of TRPV4 shows an interaction with osmolarity: namely, increased thermal responses in hypo-osmotic conditions and a diminished response in hyper-osmotic solutions (Guler *et al.*, 2002).

4. Structure–Function Relationships

The important sites for heat activation and binding of ligands such as 4α -PDD and 5',6'-EET have yet to be fully elucidated. A YS motif in the TM2-TM3 linker domain of TRPV1 is involved in the binding of capsaicin (Jordt and Julius, 2002) and this motif (Y555, S556) is found at the cytoplasmic interface of TM3 in TRPV4. Substitution of the tyrosine by alanine (Y555A) essentially abolished the Ca²⁺ and current responses to heat and 4α -PDD, whereas the more conservative substitution by phenylanaine (Y555F) is without effect (Vriens *et al.*, 2004b). The Y555A mutant shows normal responses to hypotonicity and arachidonic acid indicating that these stimuli activate TRPV4 by a different mechanism.

Activation of TRPV4 channel activity involves a calcium mediated potentiation step. Human TRPV4 shows a basal activity that is dependent on a low level of Ca^{2+} influx, presumably through the activated channels. This background activity is lost when external Ca^{2+} was removed but can be restored by liberation of Ca from intracellular stores with thapsigargin (Strotmann *et al.*, 2003). This calcium sensitivity suggests that TRPV4 activation requires coincident activation by calcium and another stimulus.

Desensitization of 4α -PDD-evoked currents is also a Ca²⁺-dependent process and removal of extracellular calcium greatly attenuates desensitization. A phenylalanine residue in TM6 is important for Ca²⁺-mediated desensitization and TRPV4 channels expressing the F707A mutation showed markedly reduced Ca²⁺-mediated desensitization (Watanabe *et al.*, 2003a). A similar critical influence of a hydrophobic amino acid in TM6 (Y671) on Ca²⁺-mediated desensitization has been noted for TRPV1 (Mohapatra *et al.*, 2003).

5. Behavioral Evidence for a Nociceptive Role for TRPV4

TRPV4 null mice show a significantly reduced behavioral response to noxious pressure applied to the tail and a reduced frequency of writhing after intra-abdominal administration of acetic acid (Suzuki *et al.*, 2003a). The reduced mechanical responsiveness is associated with a higher mechanical threshold for action potential generation in the peripheral nerve. Furthermore, C-fiber responses to noxious pressure are seen in wild type but not in TRPV4–/– mice (Suzuki *et al.*, 2003a). In contrast to the reduction in noxious mechanical responses, the TRPV4 knockout mice display normal responses to noxious heat and low threshold mechanical stimulation with

von Frey hairs (Suzuki et al., 2003a; Todaka et al., 2004). Despite the absence of any behavioral change to heat stimuli, a more detailed examination of the neuronal firing evoked by heat has revealed a deficit in TRPV4 null mice (Todaka et al., 2004). Neuronal firing is evoked with skin temperatures above 33°C in normal mice, while the threshold temperature is raised to 40°C in the TRPV4 null mice. However, both wild type and TRPV4 null mice show robust increases in firing when the skin temperature is raised to 50° C. A difference in behavior in the hot plate test between wild type and TRPV4 null mice has been noted at early times (20 mins) after carrageenan administration. However, this effect is dependent on the hot plate temperature. In this study, clear differences in withdrawal latency were noted with moderate temperatures (40-42°C) but no difference was seen with a 50°C challenge, which is the typical temperature used in this test. Overall, the data indicate a deficit in thermal responses at warm/low noxious temperatures in TRPV4 null mice, whereas the thermal responses are normal at higher noxious temperatures. This pattern of responses is consistent with a role in sensing warm temperatures.

Exposure to hypotonic saline evokes a depolarizing response in some capsaicin-sensitive DRG neurons. About 50% of the neurons that respond to hypotonicity are also activated by the TRPV4 agonist, 4a-PDD (Alessandri-Haber et al., 2003). Intradermal injection of hypotonic solution evokes action potentials in about 50% of cutaneous C-fibers but this stimulus does not evoke any obvious flinching behavior. The C-fiber nerve activity can be augmented by prior injection of the sensitizing agent PGE₂ and, under such conditions, administration of hypotonic saline now evokes a clear nociceptive response (Alessandri-Haber et al., 2003). A role for TRPV4 in this response has been demonstrated in experiments where intrathecal administration of antisense oligonucleotides directed against TRPV4 reduced the TRPV4 protein levels in the saphenous nerve by 41% and significantly reduced the behavioral effects of the hypotonic saline challenge by 40-50% in normal (Alessandri-Haber et al., 2003) and taxol treated rats (Alessandri-Haber et al., 2004). TRPV4 antisense oligonucleotides treatment also abolished the mechanical hyperalgesia induced by taxol treatment (Alessandri-Haber et al., 2004) but had no obvious effect on the noxious mechanical or thermal responses in normal rats (Alessandri-Haber et al., 2003). These data indicate a role for TRPV4 in nociception in pathological conditions.

C. The Roles of TRPV3 and TRPV4 in Warm Responses of Native Cells

The heat evoked calcium responses, whole cell currents, and reversal potential and single channel conductance in freshly isolated mouse aortic



FIGURE 4 (A) Current response of TRPV4 to increasing temperature. (Reprinted from Guler *et al.*, 2002.) (B) Temperature evoked increases in intracellular calcium concentration in response to step changes in temperature in mouse 308 keratinocytes. Reprinted from Chung *et al.* (2003).

endothelial cells, which express TRPV4, are similar to those seen for heterologously expressed TRPV4 (Watanabe *et al.*, 2002b). Heat-activated calcium responses and cationic membrane currents have also been found in the mouse 308-keratinocyte cell line (Chung *et al.*, 2003), which expresses TRPV3 as well as TRPV4. These keratinocyte cells exhibit heat activated calcium responses and whole cell currents with a threshold temperature of $32 \,^\circ$ C (Fig. 4B). The heat-activated Ca²⁺ responses in this cell line resemble those of TRPV4 rather than TRPV3. They are augmented by hypotonic conditions or 4α -PDD and reduced by hypertonicity (Gao *et al.*, 2003). Furthermore, the single channel properties show the hallmarks of TRPV4. Thus, heat and 4α -PDD activate a ~150 pS conductance channel in cell attached patches and the single channel response to heat, but not 4α -PDD, is lost after patch excision.

Cultured keratinocytes from mice respond to a heat stimulus with a threshold temperature of about 32°C (Chung *et al.*, 2004b). Although primary cultures of keratinocytes express both TRPV3 and TRPV4 the available evidence suggests that the majority of the responses have similar properties to TRPV4 currents, while a small percentage of responses resemble those reported for TRPV3. Like TRPV4 currents, the majority of the heat evoked cationic currents desensitize with repeated heat challenges and the TRPV4 agonist 4α -PDD evokes large currents in these cells.

The responses do, however, show some difference to TRPV4 expressed in HEK293 cells. First hypotonic solutions fail to elicit a response, although the heat response is augmented when the tonicity is reduced. Second, ruthenium red inhibits the inward current but, uniquely, activates an outward current in keratinocytes. Heat and/or hypotonicity fail to evoke currents in keratinocytes from TRPV4 null mice, consistent with a major role for TRPV4 in heat responsiveness. One additional possibility is that TRPV3 and TRPV4 can form hetero-oligomers. This suggestion is given some support by the finding that ruthenium red evokes slow outward currents in cells heterologously expressing TRPV3 [M. Chung and M. Caterina, unpublished data cited in Chung *et al.*, (2004b)].

A minority of mouse keratinocytes ($\sim 2.5\%$) display a heat-evoked current that resembles TRPV3 mediated responses (Chung *et al.*, 2004b). These currents exhibit a strong rectification and, like TRPV3, the responses are sensitized rather than desensitized by repeated heat challenges (Peier *et al.*, 2002b; Chung *et al.*, 2004b).

Although TRPV3 is expressed in nearly all keratinocytes, few express TRPV3-like currents. As most of the TRPV3 protein detected by immunostaining is intracellular it is possible that the expression level at the cell surface is normally insufficient to detect a TRPV3 mediated heat-evoked current. Alternatively, functional expression may be inhibited by some endogenous mechanism present in keratinocytes. Whatever the reason, overexpression of TRPV3 in keratinocytes results in TRPV3-like responses, so any suppressive factors can be overcome with sufficiently high expression levels.

VI. COLD ACTIVATED ION CHANNELS

Some sensory neurons are stimulated by cooling and these can be divided into two categories on the basis of their threshold temperatures. One subset contains A δ - and C-fibers that are generally mechanically insensitive. These neurons are activated by mild cooling with a threshold temperature of about 30°C and by the cooling agent menthol (Dubner *et al.*, 1975; Darian-Smith, 1984; Campero *et al.*, 2001). The other group, the cold nociceptors, are generally high-threshold mechanosensitive neurons that are activated by much lower temperatures, <20°C (Campero *et al.*, 1996)

A number of ion channel mechanism have been proposed to explain cold sensitivity, including an inhibition of potassium channels, such as TREK-1 (Maingret *et al.*, 2000; Reid and Flonta, 2001a; Viana *et al.*, 2002) and an activation of Na channels, such as ENaC (Askwith *et al.*, 2001). Clearly the overall firing of a nerve will depend on the balance of channel activities and will depend on the temperature sensitivities of non-TRP channels. The relative contributions and importance of different mechanisms may

also vary under different conditions. However, there is persuasive evidence that the opening of specific TRP channels codes for lowered temperatures in sensory neurons and that TRPM8 is a specific receptor for cooling.

A. TRPM8 is Activated by Cool Temperatures and Menthol

Although early investigations failed to detect a specific conductance increased by cold, the finding that cooling increased the intracellular Ca^{2+} level in cultured DRG neurons by a mechanism that was dependent on external calcium and independent of external Na⁺, suggested a cold activated current (Gotoh *et al.*, 1998). This was shown directly in electrophysiology experiments on DRG neurons (Reid and Flonta, 2001b) and has now been confirmed in several laboratories. Cooling activates a current response (Fig. 5A) associated with a conductance increase in a subset of sensory



FIGURE 5 (A, B) Currents evoked by cooling TRPM8 expressing CHO cells. (A) Cooling temperature ramp and current response. (B) Plot of current amplitude against temperature. (C) Currents in a rat DRG neuron evoked by cooling under normal conditions and in the presence of 10 and 100 μ M menthol, showing the increased threshold temperature with menthol. (D) Response of TRPM8 expressing CHO cell in the presence and absence of menthol showing a similar increase in temperature threshold.

neurons with a temperature threshold of about 27-30°C (Reid and Flonta, 2001b; McKemy *et al.*, 2002; Okazawa *et al.*, 2002; Reid *et al.*, 2002; Nealen *et al.*, 2003). The activated cation permeable channels allow both Na⁺ and Ca²⁺ to enter the cells (Reid *et al.*, 2002).

The molecular correlate of this cool activated current is TRPM8, which was identified and characterized independently in two laboratories (McKemy *et al.*, 2002; Peier *et al.*, 2002a).

1. TRPM8 Expression in Sensory Neurons

TRPM8 is a 1104 amino acid channel, which has been shown by both in situ hybridization and functional studies to be expressed in 5-12% of adult sensory neurons with expression restricted to the smaller diameter neurons (18–19 µm for DRG and 21 µm for trigeminal neurons) (McKemy et al., 2002; Reid et al., 2002; Peier et al., 2002a; Nealen et al., 2003). Co-labeling with other markers has illustrated that TRPM8 expressing neurons do not stain with neurofilament antibodies and, under normal conditions, TRPM8 is generally not found in neurons expressing the capsaicin-sensitive channel TRPV1, the neuropeptide CGRP or the binding site for IB4 (Peier et al., 2002a; Nealen et al., 2003; Thut et al., 2003). These data indicate that TRPM8 is expressed in a subpopulation of small diameter neurons distinct from the TRV1 expressing polymodal nociceptors. TRPM8 containing cells also express the high-affinity NGF receptor TrkA, and TRPM8 is not found in sensory neurons in TrkA null mice (Peier et al., 2002a). Single cell PCR has also revealed that TRPM8 mRNA is detectable in a significantly larger proportion of cool sensitive than cool insensitive neurons (Nealen et al., 2003). An overlap in TRPM8 expression with TRPV1 has been noted when sensory neurons are cultured together with high levels of NGF, even for a short time (ca 24 h), perhaps indicating upregulation of TRPM8 and/or TRPV1 in the other neuronal subpopulation (Story et al., 2003). This finding may well underlie the observations of several groups that a significant number of TRPM8 expressing neurons in culture are also capsaicin sensitive (McKemy et al., 2002; Reid et al., 2002).

2. Activation by Cooling

Heterologously expressed TRPM8 is not responsive to capsaicin or hypotonic medium but is activated by cooling with a threshold temperature of about 23–26°C (Fig. 5A, B) (McKemy *et al.*, 2002; Peier *et al.*, 2002a). Unlike TRPV1 and other TRPV channels, TRPM8 is not blocked by ruthenium red. However, TRPM8 is blocked by several TRPV1 antagonists, for example BCTC, $EC_{50} = 0.8 \mu M$ and capsazepine, $EC_{50} = 18 \mu M$ (Behrendt *et al.*, 2004). In agreement with this finding 50- μM capsazepine almost completely blocked cold-evoked currents in DRG neurons and a similar inhibitory effect was noted for the store operated calcium channel blocker SKF96365 (Reid *et al.*, 2002).

Long-term exposure (2–4 days) of DRG neurons to the neurotrophin NGF has been reported to influence the cold evoked current and subsequent Ca^{2+} response. NGF raised the threshold temperature for activation by about 3°C and significantly increased the amplitude of the cold-evoked responses (Reid *et al.*, 2002).

3. Chemical Activators

TRPM8 is activated by some chemicals, such as menthol and icilin, which evoke a cool sensation in behavioral experiments. These agents raise the thermal threshold for activation to higher temperature in both heterologous expression systems (Fig. 5D) and in isolated DRG neurons (Fig. 5C) (McKemy *et al.*, 2002; Reid *et al.*, 2002; Peier *et al.*, 2002a; Brauchi *et al.*, 2004). In this way TRPM8 is activated at body (e.g., buccal) temperatures and conveys a cool sensation.

TRPM8 is a nonselective cation channel with relative permeabilities of $P_{\rm Cs}/P_{\rm Na} = 1.1-1.4$, $P_{\rm K}/P_{\rm Na} = 1.2-1.3$ and an appreciable calcium permeability $P_{\rm Ca}/P_{\rm Na} = 1-3.3$ (McKemy *et al.*, 2002; Peier *et al.*, 2002a), very similar to the values for cold activated channels in trigeminal neurons: $P_{\rm Cs}/P_{\rm Na} = 1-1.2$, $P_{\rm K}/P_{\rm Na} = 1-1.1$, and $P_{\rm Ca}/P_{\rm Na} = 3.2-8.4$ (McKemy *et al.*, 2002; Okazawa *et al.*, 2002).

TRPM8 mediated currents show a calcium dependent desensitization, which in DRG neurons is associated with an increase in the threshold temperature of about 2.5 °C. This desensitization requires a rise in intracellular calcium and can be reduced by strong intracellular calcium buffering (Okazawa *et al.*, 2002; Reid *et al.*, 2002). Activation of TRPV1, which like TRPM8 is a calcium permeable channel that undergoes a calcium-dependent desensitization, leads to a cross desensitization of TRPM8 in those DRG neurons that show dual sensitivity to cold and capsaicin (Reid *et al.*, 2002). This result suggests a spatial proximity between the channel subtypes sufficient for calcium influx through TRPV1 to desensitize adjacent TRPM8 channels.

4. Mechanisms of Activation

Several differences in the mechanism of TRPM8 activation by menthol and icilin have been noted. Icilin-evoked, but not menthol-evoked, responses occur after a significant latency and require the coincident action of intracellular Ca^{2+} , which can be supplied either by calcium release from intracellular stores or by calcium permeating TRPM8 (Andersson *et al.*, 2004; Chuang *et al.*, 2004). Furthermore, icilin-evoked currents are sensitive to changes in the intracellular pH. For example, they are inhibited by mild intracellular acidification and augmented by alkalinization with a pH₅₀ of 7.2. In contrast, menthol responses are relatively pH insensitive (Andersson *et al.*, 2004; Behrendt *et al.*, 2004). Following the approach pioneered for TRPV1, the Julius group has used species differences in chemosensitivity—here the lack of icilin sensitivity of chicken TRPM8—to identify putative ligand binding sites. Transfer of TM2 and TM3 regions from the rat to chicken TRPM8 was sufficient to confer icilin sensitivity and of the five amino acid differences in this region only one amino acid was important to confer this chemosensitivity. A single substitution of a glycine residue with alanine at position 805 in the rat channel led to a loss of icilin-sensitivity whereas the substitution of the corresponding alanine residue in chicken TRPM8 with glycine conferred icilin sensitivity. In addition, two further amino acids located nearby at positions N799 and D802 also contribute to the ability of icilin to activate TRPM8 (Chuang *et al.*, 2004).

5. Noxious Cold Responses

A subset of rat DRG neurons (4–13%) are activated at lower temperatures than the cool receptors with threshold temperatures of ~15–20°C (Nealen *et al.*, 2003; Story *et al.*, 2003; Thut *et al.*, 2003; Djouhri *et al.*, 2004). These neurons are also sensitive to capsaicin, indicating that they are polymodal nociceptors (Story *et al.*, 2003). This finding is consistent with reports that some afferent fibers activated by cold temperatures also respond to noxious heat (Leem *et al.*, 1993; Campero *et al.*, 1996; Koltzenburg *et al.*, 1997), and that treatment with the ultrapotent TRPV1 agonist, resiniferatoxin, leads to a reduction in responsiveness to both noxious heat and noxious cold (Hama, 2002).

B. TRPA1 as a Noxious Cold Receptor

A candidate molecule for transduction of lower temperatures is TRPA1 (originally termed ANKTM1) (Story *et al.*, 2003). This TRP channel is composed of ~1120 amino acid residues, with 14 predicted N-terminal ankyrin like repeats. TRPA1 is expressed in a small percentage (3.6%) of mouse DRG neurons almost all of which (~97%) coexpress the neuropeptide CGRP and TRPV1. A higher percentage (20%) of neonatal rat trigeminal neurons express TRPA1 (Jordt *et al.*, 2004). The smaller percentage of TRPA1 positive neurons is reflected in the finding that overall about 30% of TRPV1 positive mouse DRG neurons coexpress TRPA1. In contrast there is no overlap in the expression of TRPA1 and TRPM8.

TRPA1 is insensitive to heating and hypotonicity. In contrast, it is activated when the temperature is lowered to below 20°C (Story *et al.*, 2003;

Bandell *et al.*, 2004), although one other study has failed to detect any response in rat TRPA1 expressing cells (Jordt *et al.*, 2004). The reported threshold temperature for heterologously expressed mouse and human TRPA1 is about 17°C, which is about 5°C lower than that required to activate TRPM8 under similar experimental conditions. TRPA1 is insensitive to menthol and capsaicin but is activated by icilin, although at higher concentrations than those required to stimulate TRPM8. Like the other thermosensitive TRP channels, it is a nonselective cation permeable channel with $P_{\rm K}/P_{\rm Na} = 1.2$, $P_{\rm Cs}/P_{\rm Na} = 1.4$, $P_{\rm Ca}/P_{\rm Na} = 0.8$, and $P_{\rm Mg}/P_{\rm Na} = 1.2$ (Story *et al.*, 2003).

1. Chemical Activators

Mustard oil has long been known to activate some polymodal nociceptors (Szolcsanyi, 1996). The basis for this chemical sensitivity appears to reside in TRPA1, which is activated by a series of isothiocyanate compounds including mustard oil (Bandell *et al.*, 2004; Jordt *et al.*, 2004) as well as other pungent agents such as cinnamaldehyde (Bandell *et al.*, 2004). Some agonists at TRPA1 (eugenol, gingerol) are also TRPV1 agonists, which suggest some molecular similarity in the binding sites despite the significant differences in amino acid sequence between these two TRP channels.

PLC linked GPCR activation (e.g., via bradykinin or muscarinic receptors) leads to TRA1 activation (Bandell *et al.*, 2004; Jordt *et al.*, 2004), and a specific PLC inhibitor, U73122, has been reported to inhibit the responses to cinnamaldehyde and to GPCR activation by bradykinin, and strongly reduce the response to cold. PLC activation can lead to increases in intracellular calcium via the IP3 pathway, and Jordt *et al.* (2004) reported that procedures that raise the calcium concentration (e.g., thapsigargin) augment the TRPA1 response, although this effect was not confirmed in the other major study in this area (Bandell *et al.*, 2004). PLC activation also generates diacylglycerol, and a cell membrane permeable analog (OAG) activates TRPA1 directly as does the DAG metabolite arachidonic acid (Bandell *et al.*, 2004). Whether these agents act directly on the TRPA1 channel is unclear as they can act on other molecular targets (Hardie, 2003).

C. Cold Hyperalgesia

There is little information on the contribution of peripheral mechanisms to cold hyperalgesia and most studies have focused on central mechanisms. This contrasts sharply with the wealth of information on peripheral mechanisms of heat and mechanical hyperalgesia. Cold hyperalgesia occurs in neuropathic conditions (Gracely *et al.*, 1992; Wahren and Torebjork, 1992;

Frost *et al.*, 1998), and, although the overall prevalence has not been well documented, it has been estimated that about 20-25% of patients with post-herpetic neuralgia have significant cold allodynia (S.N. Raja, personal communication). Cold hyperalgesia is similarly seen after experimental nerve injury in rats (Bennett and Xie, 1988; Choi *et al.*, 1994; Jasmin *et al.*, 1998; Sato *et al.*, 2000). A cold hypersensitivity has also been noted in DRG neurons isolated from neuropathic rats following spinal nerve ligation, using calcium imaging to detect cold sensitive neurons (Djouhri *et al.*, 2004). Here the percentage of neurons showing either low (~16°C) or high (~23°C) threshold cold responses was increased in the uninjured L4 ganglion but not in the injured L5 ganglion.

In general, there are little data on cold hyperalgesia in inflammatory conditions. Cold and damp weather conditions have been reported to exacerbate the pain in rheumatoid arthritis but the evidence for a direct link to ambient temperature is weak (Jamison et al., 1995). An antinociceptive effect of cold has been reported during early inflammation induced by complete Freund's adjuvant in animals (Jasmin et al., 1998). Conversely, hyperalgesia to noxious cold ($<10^{\circ}$ C) has been noted at later times (>1 week) of inflammation at a time when heat and mechanical hyperalgesia are diminishing (Jasmin et al., 1998; Takahashi et al., 2003). This has been demonstrated by both behavioral studies and single nerve fiber recordings. Rats with local inflammation showed an increased response (paw shaking) to a reduced temperature (25°C) and an increased percentage of nerve fibers that responded to cool temperatures without any obvious change in the thermal threshold (~26-27°C). Inflammation also resulted in an increase in the percentage of nerve fibers responding to noxious cold. This increase appeared to be due to an increase in the number of individual fibers responsive to mechanical, heat, and cold stimuli and a reduction in the mechano-heat population, perhaps indicating a gain of cold sensitivity in some neurons (Takahashi et al., 2003; Mizumura et al., 2004). The relationship between this cold hyperalgesia and TRP channel expression is unclear. In the only published study to date, RT-PCR analysis revealed no changes in expression of either TRPM8 or TRPA1 mRNA at time points that correlated with inflammatory cold hyperalgesia (Mizumura et al., 2004).

VII. TRP CHANNELS AS INVERTEBRATE THERMOSENSORS

Invertebrates such as *Caenorhabditis elegans* and *Drosophila melanogaster* show clear physiological and behavioral responses to temperature changes. A homolog of TRPA1 in *Drosophila*, termed *painless*, is expressed in the thermosensory neurons and has been shown to be important for responses to

7. TRP Channels as Thermosensors

noxious heat as well as mechanical stimuli. Although the exact role of this protein in mechanotransduction is unclear, the finding that the *Drosophila* ortholog of TRPA1 (dANKTM1) confers heat sensitivity rather than cold when heterologously expressed suggests that this channel has a direct role in heat thermosensitivity (Viswanath *et al.*, 2003). dANKTM1, which shows a 54% similarity to mouse TRPA1, is activated at a threshold temperature of 25–29°C close to the preferred environmental temperature for *D. melanogaster* of 24°C. The finding that the mammalian channel is cold activated while the invertebrate channel is warm activated may help in mapping the temperature sensing domains of these TRP channels.

Other non-TRP channels may also play a role in invertebrate thermosensitivity. In *C. elegans*, a cyclic nucleotide-gated channel (TAX-2/TAX-4) that is highly homologous to the olfactory and photoreceptor channels in vertebrates is required for thermosensation, as well as for taste and olfaction (Mori, 1999).

VIII. CONCLUSIONS

The cloning and expression of TRP channels has revealed that TRPV1–4, TRPM8, and TRPA1 are thermosensitive with sensitivities spanning the relevant range of environmental and physiological temperatures. Furthermore, their expression patterns are consistent with major roles as thermosensors. Some of these TRP channels are clearly sensitive to non-thermal stimuli and therefore can act as polymodal receptors in sensory nerve cells and in other tissues.

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CHAPTER 8

Acid Sensing Ionic Channels

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I. OVERVIEW

Tissue acidosis is a mediator of pain and accompanies inflammation and ischemia. Two families of cation channels in sensory nerve endings that are activated when the extracellular pH becomes acidic (TRP and ASICs) apear good candidates for the acid sensor that links acidosis to nociceptor action potential discharge. We will focus here on acid sensing ionic channels (ASICs), a family of cation channels that are expressed in many types of sensory neurons. All ASICs are permeable to Na⁺, some also conduct K⁺ and Ca²⁺. ASIC1 and ASIC3 are highly acid sensitive and open transiently when the extracellular pH drops only slightly below neutral. ASIC activity is modulated by neuropeptides, extracellular Ca^{2+} , associated proteins, and protein phosphorylation. While there is no definite proof of a role of ASICs in nociception as for yet, it appears likely that ASICs play a role in cardiac and muscular nociception. Because of the homology of ASICs with mechanosensitive cation channels from the nematode C. elegans, a role of ASICs in mechanosensation was proposed. While some initial data suggested such a role, recent studies rather propose no or only a minor role of ASICs in mechanosensation.

II. INTRODUCTION

This chapter will discuss the structure of ASICs, their response profiles to acid, localization in a subset of sensory neurons, possible roles in mediating pathological pain, and results from knockout experiments.

Acids have long been known to cause severe pain when applied accidentally to skin lesions or mucous membrane (Keele and Armstrong, 1964). Pronounced acidification also occurs in inflammation or ischemia when the local pH drops to values as low as 5.4 (Häbler, 1929; Jacobus et al., 1977). The intracellular pH decreases to 6.2 within 2 min during exhausting muscle contractions (Pan et al., 1988). In ischemia as occurs in inflamed tissue or working skeletal muscle, cells switch to anaerobic glycolysis due to an imbalance of perfusion and massive increase in local metabolism. Lactate/H⁺ cotransporters actively extrude lactic acid from the cell, for example, in muscle (Geers and Gros, 2000) and leucocytes (McCarty et al., 1966). Lactatic acid extrusion contributes together with other mechanisms, such as transmembrane CO₂ diffusion and Na⁺/H⁺ exchangers, to the acidification of the cellular environment. Such acidification can cause pain sensations. Intracutaneous injections of buffers with a pH of 6.2 were painful as were applications onto blister bases (Keele and Armstrong, 1964). In these studies pain sensation was transient, which can be explained by the high-buffering capacity of the tissue and the fast removal of protons by the blood stream. However, the transient nature of acid-evoked pain may also partially result from transient acid-evoked ionic currents through ASICs (see later). Pain was also evoked by CO_2 pulses applied to the nasal mucosa (Kobal and Hummel, 1990; Anton *et al.*, 1992). A more persistent pain was observed on intracutaneous pressure injection of acidic buffers (pH 5.2), and this was accompanied by hyperalgesia to punctuate mechanical stimuli (Steen and Reeh, 1993). pH effects on nociceptors match the psychophysical observations (Steen *et al.*, 1999). Corneal nociceptors were driven by acetic acid, and cutaneous nociceptors develop discharge responses at pH values between 6.1 and 6.9 (Belmonte *et al.*, 1991; Steen *et al.*, 1992).

III. NATIVE PROTON-GATED CATION CHANNELS IN SENSORY NEURONS

Regarding the mechanism of pH-induced nociceptor excitation, the first evidence came from electrophysiological studies on dorsal root ganglion (DRG) neurons. Two different types of proton-gated inward currents were observed (Fig. 1). In most cells, protons induced a fast sodium inward



FIGURE 1 Native proton-induced currents in sensory neurons. (A) Different types of inward currents induced by rapid application of extracellular acid (pH 6.1) to sensory neurons from rat DRG. The fast inactivating component fits well with activation of ASICs, whereas the sustained current likely is due to activation of TRP channels like TRPV1. (B) Sustained responses of primary afferent nociceptors to rapid application of extracellular acid (pH 6.1, n = 13).

current with activation starting at pH 7.0, a maximum current at around pH 6.0 and an inactivation time constant of 0.5 s (Krishtal and Pidoplichko, 1980; Konnerth et al., 1987; Davies et al., 1988; Morad and Gallewaert, 1989). A second pH-induced cation conductance was found only in capsaicin sensitive DRG neurons that activated at lower pH values (below pH 6.2) and exhibited a slower rate of inactivation (Bevan and Yeats, 1991; Zeilhofer et al., 1996, 1997). Acidic solutions of pH < 6.2 evoked a sustained, slowly inactivating inward current in neurons voltage clamped at negative holding potentials. The size of the currents increased with increasing proton concentrations, and the response was restricted to a subpopulation (approximately 45%) of adult and neonatal rat DRG neurons and was distinct from a rapidly activating and inactivating proton-induced inward sodium current that was also found in DRG neurons. The sustained proton-activated current was due to an increase in cation conductance that allowed K^+ , Cs^+ , and Na⁺ to pass with PK⁺/PNa⁺ = 1.32 and PCs⁺/PNa⁺ = 1.12. Radioactive ion efflux experiments performed on neonatal rat cultured DRG neurons showed that protons also increased the permeability to both [¹⁴C]guanidinium and ⁸⁶Rb⁺ ions (Bevan and Yeats, 1991). The half-maximal increase in efflux rate for ⁸⁶Rb⁺ occurred at pH 5.8. Acidic solution also stimulated the efflux of ⁸⁶Rb⁺ in cultures of adult rat sensory neurons. Neurons that showed a late, sustained proton-activated current also responded to capsaicin. In addition, no proton-activated fluxes of either [¹⁴C]guanidinium or ⁸⁶Rb⁺ ions were observed in cultures of DRG neurons that had been treated with high concentrations of capsaicin (10 µM) to kill the capsaicin-sensitive neurons (Bevan and Yeats, 1991; Bevan and Geppetti, 1994).

Slowly inactivating proton-induced currents were accompanied by a rise of the intracellular Ca^{2+} concentration that was dependent on the presence of extracellular Ca^{2+} . With 1.6 mM extracellular Ca^{2+} and at a holding potential of -80 mV fractional calcium permeability of capsaicin-induced currents (at pH 7.3) was 4.30 +/- 0.17% and of slowly inactivating proton-induced currents (at pH 5.1) was $1.65 \pm -0.11\%$. The calcium permeability of fast inactivating proton-induced currents was negligible. Fractional calcium permeability of slowly inactivating proton-induced currents increased with increasing extracellular Ca²⁺ concentration (0.5-4.8 mM) and decreased linearly with decreasing extracellular pH by about 0.7% per pH unit over the pH range investigated. When determined at the same extracellular pH fractional calcium permeabilities were significantly different for the two current types at all pH values tested (Zeilhofer et al., 1996, 1997). On repeated activation, the slowly inactivating current type exhibited strong tachyphylaxis (Petersen and LaMotte, 1993), which was prevented by increasing intracellular calcium buffering capacity or inhibition of calcium-dependent phosphatase 2B (calcineurin) (Cholewinski et al., 1993; Docherty et al., 1996).

8. ASCIs

Whereas the slow conductance shares many similarities with the pH-induced current trough TRPV1 channels (Chapter 6), the fast pH-induced current resembles the currents that are carried by ASIC type ion channels found in many species including frog and chicken (Davies *et al.*, 1988; Akaike *et al.*, 1990; Kress and Zeilhofer, 1999).

IV. CLONED ASICs

The molecular identity of H^+ -gated cation channels in mammalian sensory neurons remained unknown until the cloning of two novel structurally unrelated families of cation channels.

1. Expression cloning of a capsaicin gated cation channel led to the identification of the capsaicin receptor VR1 (TRPV1) (Caterina *et al.*, 1997), a member of the TRP family of ion channels. TRPV1 was shown to be activated and modulated by a multitude of nociceptive signals including extracellular acidosis and heat (Tominaga *et al.*, 1998).

2. Cloning of homologs of the *C. elegans* degenerins, a family of ion channels involved in neurodegeneration and mechanosensation of the nematode (Ernstrom and Chalfie, 2002), led to the identification of the ASICs (Waldmann and Lazdunski, 1998; Kress and Zeilhofer, 1999; Waldmann, 2001; Krishtal, 2003), a family of neuronal ion channels that are activated by a drop of the extracellular pH.

We will focus here mainly on ASICs, since TRP channels are the topic of several other chapters in this book.

ASICs belong to the epithelial amiloride sensitive Na⁺ channel/degenerin (ENaC/DEG) family of ion channels (Fig. 2). The ENaC/DEG family of ion channels has members in all sequenced metazoan species (nematodes, insects, ascidians, snails, . . .), there are no members of this ion channel family in yeast. ENaC/DEG channels are proteins of about 50–70 kDa. With two transmembrane domains and a large cysteine-rich extracellular loop, they have the same structure as the ionotropic purinergic receptors P2X (Chapter 11) (North, 1996). However, they lack significant sequence homology, and this suggests that ionotropic purinergic receptors and ASICs do not share a common ancestor. To date, the ENaC/DEG family has nine members in humans. There are two major phylogenetic branches (Fig. 2): (1) the constitutively active amiloride sensitive Na⁺ channel subunits (aENaC, β ENaC, γ ENaC, δ ENaC) that are mainly expressed in epithelia (Kellenberger and Schild, 2002). A heteromeric channel formed by $\alpha\beta\gamma$ ENaC is crucial for epithelial Na⁺ reabsorption and (2) the second branch



FIGURE 2 Epithelial Na⁺ channel/degenerin family of ion channels. Left: Phyllogenenetic tree indicating the members of this ion channel family in nematodes (*C. elegans*), insects (*Drosophyla melanogaster*), and mammals (human). Both insects and nematodes have about two times more members of this ion channel family than mammals. Sequences were aligned with the ClustalW program and the tree calculated with Kimura's correction for multiple substitutions. Right: The proposed transmembrane topology of this ion channel superfamily is: two transmembrane domains, a long extracellular loop, and intracellular NH₂ and COOH terminus. Two splice variants: the approximate position of the splice junction, where ASIC1a and ASIC1b or ASIC2a and ASIC2b differ in their NH2 terminus is indicated with an (*).

comprises the ASICs and brain liver intestine Na⁺ channel (BLINaC). We will focus here on ASIC1 (ACCN2), ASIC2 (ACCN1), and ASIC3 (ACCN3), since for both ASIC4 (ACCN4) (Akopian *et al.*, 2000; Grunder *et al.*, 2000) and BLINaC (ACCN5) (Schaefer *et al.*, 2000; Sakai *et al.*, 2004) neither alone nor coexpressed with other ASIC subunits an activation by extracellular acidification or an expression in sensory neurons have been reported as for yet.

Currently, five ASIC subunits have been shown to be part of homomultimeric or heteromultimeric H⁺-gated channels. Two splice variants were characterized for ASIC1 (ASIC1a, ASIC1b) and ASIC2 (ASIC2a, ASIC2b) that differ in the NH₂-terminus, the first transmembrane domain and part of the extracellular loop (Fig. 2). For ASIC3, only one transcript has been

8. ASCIs

functionally expressed in a heterologous expression system. Activation of ASICs is mediated by extracellular acidification (Waldmann *et al.*, 1997b). No activation or modulation by intracellular pH changes was reported as for yet. With acidification within a physiological relevant pH range as the only agonist ASICs desensitize and seem to be more suitable for the sensing of pH fluctuations than of sustained pH changes. However, a small sustained activity during prolonged acidosis (de Weille *et al.*, 1998; Deval *et al.*, 2003; Hesselager *et al.*, 2004) or even at neutral pH (de Weille *et al.*, 1998) was reported for ASICs. All ASICs are Na⁺ permeable. Unless mentioned otherwise below, Na⁺ is the principal physiologically relevant permeable ion, and in general, the single channel Na⁺ conductance of ASICs is between 10 and 15 pS.

V. PROPERTIES OF CLONED ASICs

A. Homomeric ASICs

The properties of homomultimeric ASICs were extensively characterized by several groups after heterologous expression in *Xenopus laevis* oocytes or mammalian cell lines (Table I, Fig. 3).

1. ASIC1a

ASIC1a, initially named ASIC1 (Waldmann et al., 1997b) or BNaC2 (Garcia-Anoveros et al., 1997), is expressed in both central and peripheral sensory neurons (Waldmann et al., 1997b). ASIC1a starts to be activated when the extracellular pH drops to below pH 6.9 (pH_{0.5} = 6.2-6.4) (Waldmann et al., 1997b; Sutherland et al., 2001). The pH dose response curve is rather steep, and ASIC1a reaches maximal activity around pH 6. The Hill coefficient for H⁺ activation is 3.24 ± 0.44 , suggesting that cooperative protonation of amino acids leads to ASIC1a channel activation (Babini et al., 2002). Thus, the acid sensitivity of ASIC1a appears well suited to sense tissue acidosis within a physiological relevant pH range. However, ASIC1a desensitizes within seconds ($\tau = 3.5$ s) and recovers only slowly ($\tau = 13$ s) (Sutherland et al., 2001) after return to neutral pH. ASIC1a is half maximally inactivated at pH 7.3 with a very steep pH resonse curve. Maximal activity and complete inactivation are only separated by 0.4 pH units (Babini et al., 2002). ASIC1a is the only ASIC for which a significant Ca^{2+} permeability was reported (pNa⁺/pCa²⁺ = 2.5) (Waldmann et al., 1997b). The Ca²⁺ permeability of ASIC1a was proposed to be a major cause of postischemic neuronal injury (Xiong et al., 2004).

	ASICIa	ASIC1b	ASIC2a	ASIC3	
				Transient	Sustained
pH ₀₅ act	6.2 (2)	6.2 (5)	4.35(1)	6.7 (4)	4.3* (9
	6.4 (4)	5.84 (6)	4.9 (5)	6.5 (8)	3.5 (8)
	6.8 (5)	5.9 (7)		6.2* (9)	
	6.56 (6)				
	5.3 (10)				
pH _{0.5} inact	7.3 (4)	7.0 (6)		7.1 (4)	4.9 (8)
	7.24 (6)			7.5* (9)	
				6.9 (8)	
τ inact (sec)	3.5 (4)	1.7 (4)	2.8 (5)	0.32(4)	
	4.0 (5)	1.3 (5)	1.65(1)		
τ rec (sec)	13 (4)	5.9 (4)	0.6 (5)	0.58 (4)	
	11 (5)	4.4 (5)		0.4 (5)	
pNa ⁺ /pK ⁺	13 (2)	2.6 (7)	10 (1)	13.5 (8)	13.5 (8)
	5.5 (4)			4.5 (4)	1.62*(9)
pCa ²⁺ /pNa ⁺	0.4 (3)	N.D.(7)	0.05 (2)	N.D. (4)	

 TABLE I

 Properties of Homomultimeric ASICs

pH_{0.5} act: pH for half-maximal activation; pH_{0.5} inact: resting pH where the channel is half-maximal inactivated; τ inact: inactivation time constant; τ rec: the time constant of channel activity recovery after return to pH values around neutral pH; pNa⁺/pK⁺ ratio of Na⁺ and K⁺ permeability; pCa²⁺/pNa⁺ ratio of Ca²⁺ and Na⁺ permeability. Most data are for rat ASICs. Values for human ASICs are marked with an (*). Note: Inactivation kinetics depends on the resting pH for most ASICs (Champigny *et al.* 1998; Hesselager *et al.* 2004). Thus, actual values will vary depending on the assay conditions. Numbers indicate: (1) Champigny *et al.* (1998); (2) Bassilana *et al.* (1997); (3) Waldmann *et al.* (1997b); (4) Sutherland *et al.* (2001); (5) Benson *et al.* (2002); (6) Babini *et al.* (2002); (7) Chen *et al.* (1998); (8) Waldmann *et al.* (1997a); (9) de Weille *et al.* (1998); and (10) Alvarez de la Roza *et al.* (2003).

2. ASIC1b

ASIC1b (ASIC β) (Chen *et al.*, 1998; Babini *et al.*, 2002) has biophysical properties that are very similar to those of ASIC1a (Table I). The main differences are: (1) ASIC1b does not conduct Ca²⁺; (2) ASIC1b requires higher acid concentration (pH_{0.5} = 5.9) than ASIC1a (pH_{0.5} = 6.4); (3) ASIC1b can be activated when the resting pH is more acidic (pH_{0.5} inact = 7.0); (4) ASIC1b has a higher K⁺ permeability than ASIC1a (pNa⁺/pK⁺ = 2.6); and (5) ASIC1b is not expressed in central neurons, the transcript of this subunit was only detected in sensory neurons (Chen *et al.*, 1998).



FIGURE 3 Properties of homomultimeric ASICs. (A) Inward currents induced by rapid application of extracellular acid in ASIC1 or ASIC2a expressing Xenopus oocytes or ASIC3 expressing COS-7 cells. Current traces were adapted from (Bassilana *et al.*, 1997) (ASIC1); (Champigny *et al.*, 1998) (ASIC2); (Waldmann *et al.*, 1997a) (ASIC3). (B) Schematic indicating pH-response curves of ASICs after a drop of the extracellular pH from physiological pH (about 7.4) to the test pH. Black indicates pH values at and below which full activity is reached, white indicates no channel activity. (C) ASICs typically desensitize when the pH is kept at acidic values. The schematic indicates the inactivation pH-response curves for a drop of the extracellular pH from varying resting pH values to a fixed acidic test pH. Black indicates the resting pH above which maximal channel activity is reached. White indicates pH values below which the channel is completely inactivated. Note that ASIC1a is partially inactivated at physiological pH and that ASIC1a, ASIC1b, and ASIC3 are virtually completely inactivated at resting pH values below pH6. The data given in Table I and the pH-response curves from the references cited in Table I were used for the schematics.

3. ASIC2a

ASIC2a (Price *et al.*, 1996; Waldmann *et al.*, 1996; Bassilana *et al.*, 1997; Garcia-Anoveros *et al.*, 1997) (MDEG, BNC1, BNaC1) starts to be activated when the extracellular pH drops to below pH 5.5, reaches half-maximal activity at pH 4.9 (Benson *et al.*, 2002) and desensitizes slower than the ASIC1 subunits. ASIC2a recovers much faster ($\tau = 0.6$ s) (Benson *et al.*, 2002) than ASIC1a or ASIC1b from inactivation after return to neutral pH (Table I). ASIC2a is expressed in central neurons. There is some controversy about the expression of ASIC2a in sensory neurons (Section VI). Because of the very high H⁺ concentrations required for ASIC2a activation, it appears unlikely that activation of homomultimeric ASIC2a by acid alone plays an important role in nociception.

4. ASIC2b

ASIC2b is expressed in both sensory and central neurons and does not generate a H^+ -gated current when expressed alone. However, ASIC2b can associate with other ASIC subunits and modulate their properties (Lingueglia *et al.*, 1997) (Section V.B).

5. ASIC3

The most acid sensitive and perhaps the most interesting ASIC for nociception is ASIC3 (DRASIC) (Waldmann et al., 1997a; de Weille et al., 1998; Ishibashi and Marumo, 1998; Babinski et al., 1999). Rat ASIC3 was only detected in sensory neurons (Waldmann et al., 1997a) while an expression of human ASIC3 was also reported in testis (Ishibashi and Marumo, 1998) and even a widespread distribution was suggested by Babinski et al. (1999). However, there are no reports about recordings of ASIC3 channels from central neurons or more generally from other cell types but sensory neurons, suggesting that functional ASIC3 channels are rather specific for sensory neurons. The ASIC3 current has two components: (1) a transient component that is highly acid sensitive ($pH_{0.5} = 6.5 - 6.7$) (Waldmann *et al.*, 1997a; Sutherland *et al.*, 2001) rapidly activating ($\tau < 5$ ms) and inactivating $(\tau = 0.32 \text{ s})$ (Sutherland *et al.*, 2001). The transient current recovers rapidly from inactivation after a return to neutral pH ($\tau = 0.58$ s) (Sutherland *et al.*, 2001). For comparison, ASIC1a requires much longer to recover ($\tau = 13$ s) (Sutherland *et al.*, 2001) and (2) a rather sustained component that requires very acidic pH (<pH 5) (Table I) for activation. The transient ASIC3 current inactivates rapidly when the resting pH is acidic. Conversely, the sustained ASIC3 current can still be activated when the pH decreases from rather acidic (<pH 6) resting pH and is also activated when the extracellular pH decreases gradually (Waldmann et al., 1997a). While rather low pH values (5.4) were reported during severe inflammation or ischemia (Häbler, 1929; Jacobus et al., 1977), there have to be doubts, whether the sustained ASIC3 current activated by acidification below pH 5 has a physiological relevance or whether this current is just a property of ASIC3 that has more biophysical than physiological interest. However, a minor (2%) sustained activation of rat ASIC3 at pH 6.3 (Deval et al., 2003) or even at neutral pH (de Weille et al., 1998) was reported. This sustained activity might be due to a "window current" at pH values where the activation and inactivation curves overlap (Benson *et al.*, 1999). That means at constant pH, the ASICs get partially activated and partially recover from inactivation leading to a small sustained activity. Since some sensory neurons, for example, cardiac sensory neurons, have huge ASIC3 currents (Sutherland et al., 2001), even a minor degree of ASIC3 activation might be sufficient to induce neuronal firing.

8. ASCIs

B. Heteromultimeric ASICs

Other members of the epithelial Na^+ channel/Degenerin family were shown to function as homo or hetero tetramers (Firsov *et al.*, 1998). Thus, it was no surprise that different ASIC subunits also assemble into heteromultimeric channels with novel properties. There were principally two approaches to understand the properties of heteromeric ASICs and their contribution to native channels in sensory neurons: (1) coexpression of different ASIC subunits, characterization of the channel properties, and comparison with those of endogenous channels in sensory neurons and (2) analysis of ASICs in sensory neurons of ASIC null mice.

With ASICs 1a, 1b, 2a, 2b, and 3, theoretically 10 different combinations of two subunits are possible. ASICs are probably tetramers just as their homologs ENaC or FaNaC (Firsov *et al.*, 1998; Coscoy *et al.*, 1999). If the stochiometry of both subunits in the terameric ion channel complex also affect ASICproperties, the number of possible heterodimeric ASICs increases to 30. In sensory neurons, several ASIC subunits coexist and might even form assemblies with three or four different subunits. Thus, the list of heteromultimeric ASICs characterized after heterologous expression given below is definitely not complete, and a greater number of heteromeric assemblies probably exist *in vivo*.

1. ASIC1a/ASIC2a

Coexpression of ASIC1a and ASIC2a resulted in a heteromultimeric H^+ -gated channel with properties between those of both subunit homomers (Bassilana *et al.*, 1997). The major change caused by coexpression is the shift in pH dependence. ASIC1a is highly acid sensitive with an activation threshold of pH 6.9. Conversely, ASIC2a with an activation threshold of pH 5.5 requires a rather high H^+ concentration for activation. Thus, the assembly with ASIC2a could decrease the activity of ASIC1a at moderately acidic pH by shifting the dose-response curve to more acidic pH. Pharmacological analysis of ASIC currents in central neurons suggests that ASIC1a/ASIC2a heteromeric channels exist *in vivo* (Baron *et al.*, 2002).

2. ASIC1b/ASIC2a

Inactivation of ASICs becomes faster at increasing H^+ concentrations (Champigny *et al.*, 1998; Hesselager *et al.*, 2004). Coexpression of ASIC1b and ASIC2a results in pH independent desensitization kinetics, suggesting the formation of heteromultimeric channels (Hesselager *et al.*, 2004).

3. ASIC2a/ASIC2b

ASIC2a has a single exponential desensitization kinetics and a very low K^+ permeability. Coexpression with ASIC2b that is not activated by H^+ when expressed alone causes alteration in channel properties (Lingueglia *et al.*, 1997) (Fig. 4): (1) The desensitization kinetics becomes biphasic with a transient and a rather sustained current component and (2) The slowly desensitizing current does not discriminate between Na⁺ and K⁺. Those profound changes in channel gating and selectivity after coexpression of the inactive ASIC2b subunit with ASIC2a strongly suggest the formation of heteromultimeric channels.



FIGURE 4 Properties of heteromultimeric ASICs. Left: acid-induced currents in ASIC2a, ASIC2b, or ASIC3 expressing COS cells at -60 mV and at +30 mV (Na⁺ equilibrium potential). Coexpression of ASIC2b with ASIC2a alters both kinetics and selectivity. Inactivation becomes biphasic, and the slowly inactivating current is outward at +30 mV indicating that it is carried by K⁺. Coexpression of ASIC2b with ASIC3 also changes the selectivity of the sustained ASIC3 current, it becomes K⁺ permeable. The current traces were adapted from Lingueglia *et al.* (1997).

4. ASIC2a/ASIC3

Coexpression resulted in increased current amplitude (activated at pH 4) and a more pronounced sustained current than with the respective homomers (Babinski *et al.*, 2000).

5. ASIC3/ASIC1a or ASIC3/ASIC1b

The formation of both heteromeric channel complexes is characterized by a loss of pH dependence of the desensitization kinetics after coexpression (Hesselager *et al.*, 2004). Since both the ASIC1 splice variants and ASIC3 are expressed in sensory neurons, those subunit combinations might be relevant *in vivo*.

6. ASIC3/ASIC2b

ASIC3 has a transient and a sustained component that are both selective for Na⁺. Coexpression of ASIC2b renders the sustained ASIC3 component permeable to Na⁺ and K⁺ (Lingueglia *et al.*, 1997). H⁺-gated cation currents with a kinetics and a selectivity resembling that of the ASIC3/ASIC2b channel were reported in sensory neurons (Bevan and Yeats, 1991; Diochot et al., 2004) suggesting that heteromultimeric ASIC3/ASIC2b channels might be relevant in vivo.

C. Pharmacology of ASICs

Except the sustained or slowly desensitizing currents of ASIC3/ ASIC2b or ASIC2a/ASIC2b heteromultimeric channels, all ASICs are blocked by the diuretic drug amiloride (K_{0.5} range 10–63 μ M) and its derivatives such as benzamile (Table II). However, amiloride at the high concentrations required for ASICs inhibition has rather broad effects on ion channels (e.g., T-type Ca²⁺ channel) or ion exchangers (Na⁺/H⁺ exchanger, Na⁺/Ca²⁺ exchanger) and is thus not a good pharmacological tool to identify specific pH-gated ion channels. Much more specific and suited for functional studies is a peptide toxin psalmotoxin (PcTx1) isolated from tarantula that blocks homomultimeric ASIC1a channels with high affinity (<1 nM) (Escoubas et al., 2000). PcTx1 does not affect the activity of other ASIC1a containing heteromultimeric ASICs and does not block other ASIC subunits. A peptide (Anthopleura elegantissima toxin 2, APETx2) that blocks the transient ASIC3 current (IC₅₀ ASIC3 = 63 nM) without affecting the sustained ASIC3 current was isolated from sea anemone (Diochot et al., 2004). However, this ASIC3 inhibitor has a lower affinity for ASIC3 associated with other ASIC subunits (K_{0.5}: ASIC3 + ASIC2b, 117 nM; ASIC3 + ASIC1a, 2 µM ASIC3 +

				ASIC3	
	ASIC1a	ASIC1b	ASIC2a	Transient	Sustained
IC ₅₀ amiloride (µM)	10 (2)	21 (3)	28 (4)	63 (1)	Small increase (1)
Ibuprofen (5)	Block	_	_	_	
Fluorbiprofen (5)	$IC_{50} = 349 \ \mu M$	-	_	-	_
Aspirin (5)	_	-	_	-	Block
Salicylic acid (5)	_	-	_	-	$IC_{50} = 260 \ \mu M$
Diclofenac (5)	-	_	_	-	$IC_{50} = 92 \ \mu M$
PcT×1 (6)	$IC_{50} = 0.9 \text{ nM}$	-	_	-	_
APET×2 (6)	-	-	_	-	$IC_{50} = 63 \text{ nM}$

TABLE IIPharmacology of ASICs

Effect of the diuretic amiloride, the NSAIDs Ibuprofen, Fluorbiprofen, Aspirin, Salicilic Acid, Diclofenac, the tarantula toxin PcTX1, and the sea anemone toxin APETx2 on ASIC activity. Numbers indicate: (1) Waldmann *et al.* (1997a); (2) Waldmann *et al.* (1997b); (3) Chen *et al.* (1998); (4) Champigny *et al.* (1998); (5) Voilley *et al.* (2001); (6) Escoubas *et al.* (2000); and (7) Diochot *et al.* (2004).

ASIC1b, 0.9 μ M; ASIC3 + ASIC2a no block). APETx2 blocks ASIC3 like currents in sensory neurons only at higher concentrations with an IC50 of 216 nM probably due to an association of ASIC3 with other ASIC subunits in sensory neurons. However, at high concentrations, APETx2 also affects K_v3.4 potassium channels (38% block at 3 μ M). Thus, both PcTX1 and APETx2 are interesting tools for studying the ASIC subunit composition in native sensory neurons. With a subnanomolar affinity, PcTX1 is clearly a valuable tool for functional studies *in vivo*, with the limitation that only homomultimeric ASICs are affected. APETx2 might turn out an interesting tool for functional studies in tissues that are innervated by sensory neurons that express homomultimeric ASIC3 such as the heart (Sutherland *et al.*, 2001).

Interesting and of potential pharmacological importance is the inhibition of ASIC1a and ASIC3 by nonsteroid anti-inflammatory drugs (NSAIDs) (Voilley *et al.*, 2001). Homomultimeric ASIC1a channels are inhibited by the NSAIDs ibuprofen and its analog flurbiprofen. The sustained components of ASIC3 and heteromultimeric ASIC3/ASIC2b channel are inhibited by salicylic acid, aspirin and diclofenac, while the transient ASIC3 current remained unaffected by NSAIDs. Voilley *et al.* (2001) also demonstrated that NSAIDs inhibit acid-induced action potential firing in sensory neurons in primary culture. Thus, inhibition of ASICs might be involved in the analgesic and anti-inflammatory effect of NSAIDs.

8. ASCIs

Gadolinium (Gd³⁺) is a rather nonspecific blocker of many types of proposedly mechanosensitive cation channels (Hamill and Martinac, 2001). In line with a potential role of ASICs in mechanosensation, a block of ASIC3 and a heteromultimeric ASIC2a/ASIC3 channel by Gd³⁺ was reported (Babinski *et al.*, 2000).

D. Modulators of ASICs

ASIC activity can be modulated by neuropeptides, extracellular divalent cations, associated proteins, protein phosphorylation, proteases, and temperature (Fig. 5).

1. Modulation of ASIC Activity by Neuropeptides

ASICs are homologs of a molluscan peptide (FMRFamide) gated Na⁺ channel. Although FMRFamide was not found in mammals, other peptides with a PQRFamide C-terminus, such as neuropeptide FF (NPFF, FLFQ-PQRFamide), neuropeptide AF (NPAF, AGEGLSSPFWSLAAPQRFamide), and neuropeptide SF (NPSF, SLAAPQRFamide), were characterized there (Panula *et al.*, 1999). NPFF, NPAF, and NPSF are processed from a common precursor and are upregulated in the spinal cord in inflammatory pain



FIGURE 5 Modulation of ASIC activity. The schematic summarizes the known regulation mechanisms of ASICs discussed in Section V.D.

models (Kontinen et al., 1997) and administration of FMRFamide or NPFF modulates pain in mammals (Panula et al., 1999). ASICs were shown to be potential physiological molecular targets for FMRFamide related neuropeptide signaling (Askwith et al., 2000; Catarsi et al., 2001; Deval et al., 2003; Xie et al., 2003; Yudin et al., 2004). Because of their rapid desensitization, ASICs did not appear well suited to sense a static long lasting acidosis that is thought to occur in inflamed or ischemic tissues. While FMRFamide and related peptides (NPFF, NPSF) in μ M concentrations do not activate ASICs at neutral pH, they slow down inactivation and/or increase the amplitude of a sustained or slowly inactivating current with ASIC1a, ASIC1b, or ASIC3 (Askwith et al., 2000; Catarsi et al., 2001; Deval et al., 2003; Xie et al., 2003) and with heteromultimeric ASICs. The sustained rat ASIC3 current amplitude is typically 2% or 5% of the transient current amplitude at pH 6.3 or pH 5, respectively (Deval et al., 2003). 100 µM NPFF or NPSF increase the sustained current about tenfold (Deval et al., 2003), leading to a drastically increased charge flow during longer lasting acidification. Half-maximal effects of peptides on ASICs were reported with 33 µM FMRFamide for ASIC1a (Askwith et al., 2000), 49 µM NPFF, NPSF, or FMRFamide for ASIC3 (Deval et al., 2003). The order of application is critical. For a maximal effect, the peptide has to be present before the extracellular pH drops (Askwith et al., 2000). Thus, modulation of ASIC activity by peptides might play an important role in the modulation of sensory neuron firing during tissue acidosis.

2. Modulation of ASIC Activity by Divalent Cations

ASIC1 and ASIC3 channel activity is modulated by extracellular Ca^{2+} . Initially, both a requirement for extracellular Ca^{2+} and an inhibition of ASIC1a by high (>5mM) Ca^{2+} concentrations were reported (Waldmann *et al.*, 1997b; de Weille *et al.*, 2001). A more detailed study of the effect revealed that increasing extracellular Ca^{2+} concentrations shift the pH response curves for both activation (ASIC1a) and inactivation (ASIC1a or ASIC1b) toward more acidic pH (Babini *et al.*, 2002). The authors reported similar effects for Mg²⁺ or the polyvalent cation spermidine.

Similar effects of extracellular Ca^{2+} were also reported for ASIC3 (Immke and McCleskey, 2003). Decreasing the extracellular Ca^{2+} concentration shifts the pH-response curve of the transient ASIC3 current toward more alkaline pH. At neutral pH a rapid decrease of the extracellular Ca^{2+} concentration is sufficient ($K_{0.5} = 12 \mu$ M) to activate the rapidly inactivating ASIC3 current without a pH change. Immke *et al.* (2003) proposed a model in which ASIC3 is blocked by extracellular Ca^{2+} and acid reliefs the Ca^{2+} block leading to channel activation. In consequence, less acidity is required for channel activation at lower Ca^{2+} concentration. The ASIC3 channel activitated at neutral pH by a low Ca^{2+} concentration desensitized rapidly, suggesting that not just the pH response curve for activation but also that for inactivation was shifted toward higher pH values. Lactate is released during ischemia and complexes Ca²⁺. The group of McCleskey demonstrated an increased acid sensitivity of ASIC3 in the presence of lactate (Immke and McCleskey, 2001). Thus, the Ca^{2+} sensitivity of ASICs might have important physiological implications in the detection of ischemic conditions by sensory neurons. However, ASIC3 desensitizes rapidly and would require either an increase in pH or in Ca^{2+} concentration (e.g., via a drop of lactate concentrations) for recovery. Lactate accumulates during ischemia, however, it is unlikely that the concentrations fluctuate enough and sufficiently fast to activate ASIC3 directly via Ca²⁺ fluctuations at constant pH. The effect of Ca^{2+} chelation (e.g., by lactate) would rather mean a fine-tuning of the dynamic working range of ASICs (Immke and McCleskey, 2001; Babini et al., 2002). Thus, in the presence of lactate small pH fluctuations around neutral pH would be sufficient to activate ASIC1a or ASIC3. However, lactate would also cause very profound inactivation of ASIC1 and ASIC3 when the tissue pH drops to pH 7 and below.

Another extracellular divalent cation, Zn^{2+} , was shown to potentiate the activity of homo- and heteromultimeric ASIC2a channels (K_{0.5} ASIC2a = 120 μ M; K_{0.5} ASIC1a/ASIC2a = 111 μ M) (Baron *et al.*, 2001). Zn²⁺ shifts the pH-response curve for channel activation to slightly less acidic pH.

3. Modulators of ASIC Activity by Associated Proteins and Protein Phosphorylation

Several labs were doing yeast two hybrid screens for associated proteins of ASICs and identified several ASIC-interacting proteins with potential physiological importance.

a. PICK1. PICK1, a PDZ domain containing protein that interacts with protein kinase C (PKC), was identified as a protein that can associate with the COOH terminal residues of both ASIC1 and ASIC2 (Duggan *et al.*, 2002; Hruska-Hageman *et al.*, 2002). No direct effect of PICK1 coexpression on ASIC1 or ASIC2 channel activity was reported as for yet. PICK1 association of ASIC1 and ASIC2 might be important for the targeting of the channel subunits into cellular subdomains. The binding of PICK1 to ASIC1a was shown to be inhibited by cAMP-dependent protein kinase (PKA) phosphorylation of the intracellular ASIC1a COOH terminal domain (Leonard *et al.*, 2003). In sensory neurons, ASIC3-like currents are increased after PKC activation, a modulation that can be reproduced in heterologous expression systems after coexpression of ASIC3 with ASIC2b and PICK1 (Deval *et al.*, 2004). ASIC2b forms a heteromultimeric channel

with ASIC3 and PICK1 might tether PKC to the channel and facilitate phosphorylation of ASIC3.

b. CIPP, PSD-95, Lin-7b. Multiple PDZ domain containing proteins were shown to interact with ASIC3 and to modulate current density after heterologous coexpression with ASIC3: CIPP, a protein known to interact with several ion channels was identified as a potential partner of ASIC3 (Anzai *et al.*, 2002). CIPP increases the ASIC3 current without significantly affecting the pH dependence. Like CIPP, Lin-7b increased the ASIC3 current amplitude while PSD-95 reduced the ASIC3 current (Hruska-Hageman *et al.*, 2004). Those ASIC3 binding proteins probably modulate the current intensity via affecting ASIC3 stability or ASIC3 membrane targeting. Besides affecting current amplitude, the role of those scaffolding proteins might be the assembly of ASIC3 together with other ion channels or receptors into microdomains.

c. *MEC-2, Stomatin.* MEC-2 is associated with the *C. elegans* degenerin channel complex and is required for mechanosensation in the nematode (Goodman *et al.*, 2002). Stomatin is about 85% homologous to MEC-2. Data suggest that stomatin can associate with ASIC3 (Price *et al.*, 2004). Coexpression of stomatin profoundly inhibited ASIC3 currents without affecting the levels of ASIC3 expressed at the membrane. Thus, stomatin seems to affect ASIC gating rather than channel trafficking.

4. Modulation of ASIC Activity by Proteases

Extracellular serine proteases shift the pH-response curves of ASIC1a activation and inactivation toward more acidic pH and accelerate recovery from inactivation by a factor of 5 (Poirot *et al.*, 2004). Protease-treated ASIC1a was resistant toward the potent ASIC1a blocking toxin PcTX1. For ASIC1b, the activation pH-response curve was also shifted toward more acidic pH, while the inactivation pH response curve moved toward more alkaline pH. The underlying mechanism is probably a proteolytic cleavage of the long extracellular loop of ASIC1a or ASIC1b. Serine proteases do not affect ASIC2a or ASIC3 activity. Serine proteases are liberated during inflammation and are thought to be physiological modulators of pain (Miller and Pemberton, 2002). Proteolysis of ASICs might adapt the dynamic working pH range of ASIC1 during the course of an inflammation.

5. Modulation of ASIC Activity by Temperature

The inactivation of ASIC1a, ASIC2a, and ASIC3 channels was modulated by temperature (Askwith *et al.*, 2001). Cooling slowed down inactivation leading to an overall increase in charge flow. This might be a mechanism to further fine-tune the ASIC activity. The physiological importance of the temperature regulation of ASICs is unknown, since none of the phenotypes of ASIC null mice (Section VII) point toward an altered nociception at cold temperatures.

VI. ASIC TRANSCRIPTS, PROTEIN, AND CURRENTS IN SENSORY NEURONS

A. ASIC Transcripts

ASIC1a, ASIC1b, ASIC2b, and ASIC3 transcripts are expressed in sensory neurons. For ASIC1b and ASIC3, sensory neurons reportedly had by far the highest mRNAs expression levels, the transcripts are virtually absent in central neurons. According to a study of J. Wood and coworkers (Chen et al., 1998): (1) ASIC1a and ASIC1b were expressed in 20-25% of L4 DRG neurons; (2) >90% of ASIC1a positive neurons were of small diameter, peripherin positive, isolectin B4 (IB4) negative, and probably nociceptive; and (3) ASIC1b was localized in both small- and large-diameter neurons, of which 70% express neurofilaments and 30% express peripherin. Peripherin is a marker for small-diameter sensory neurons, Substance P and IB4 are markers for peptidergic and nonpeptidergic nociceptive C-fibers, respectively (Chen et al., 1998). The group of M. Lazdunski reported a somewhat different distribution for ASIC1 in L4-L5 DRGs (Voilley et al., 2001): both ASIC1a and ASIC1b transcripts were detected in about half of the substance P positive and half of the IB4 positive neurons. Taken together, both studies suggest that ASIC transcripts are expressed in nociceptive neurons. In the same study, Voilley et al. (2001) reported a similar distribution for ASIC2b and ASIC3 and a 6–15 fold increase in expression of all ASIC transcripts in inflammation. The increased ASIC transcription appears to be principally mediated by a mix of proinflammatory mediators such as nerve growth factor, serotonin, interleukin-1, and bradykinin (Mamet et al., 2003). Increased ASIC3 protein immunoreactivity was reported in inflamed human intestine (Yiangou et al., 2001). Conversely, the expression level of TRPV1, which was shown to be important for the development of inflammatory thermal hyperalgesia (Davis et al., 2000), was not upregulated during inflammation (Voilley et al., 2001).

The ASIC2a mRNA seems to be only expressed at very low levels in sensory neurons. *In situ* hybridizations on sensory neurons or DRGs with ASIC2a specific probes resulted either in no detection of the transcript (Lingueglia *et al.*, 1997; Voilley *et al.*, 2001) or rather weak signals (Garcia-Anoveros *et al.*, 1997; Price *et al.*, 2000; Garcia-Anoveros *et al.*, 2001). Thus, ASIC2b appears to be the predominant ASIC2 transcript in sensory neurons.

B. ASIC Immunoreactivity

Immunohistochemistry of ASIC subunits in DRGs or sensory nerve endings were reported by several groups. ASIC1a immunoreactivity apparently colocalized with substance P and calcitonin gene-related peptide (CGRP) in small-capsaicine sensitive sensory neurons (Olson et al., 1998), a distribution that fits well with that of the transcript (Chen et al., 1998; Voilley et al., 2001). Price et al. (2000) performed immunochemistry with an antibody that recognizes ASIC2a and ASIC2b and showed ASIC2 immunoreactivity in fibers surrounding guard hair follicles, the palisades of lanceolate nerve endings. Thus, ASIC2 appeared to be well placed to function as a mechanosensitive cation channel that senses hair movements. While the ASIC2a transcript was at low abundance, Garcia-Anoveros et al. (2001) localized ASIC2a immunoreactivity in the peripheral nervous system with an antibody directed against the ASIC2a NH₂ terminus. They found ASIC2a immunoreactivity in most large DRG neurons, potentially resembling low-threshold mechanosensors. In the skin, they reported ASIC2a immunoreactivity in Meissner, Merkel, penicillate, reticular, lanceolate, and hair follicle palisade nerve fiber associated corpuscles, which seems consistent with a role of ASIC2a in mechanosensing. Retrograde labeling of sensory neurons inervating the gastrocnemius-soleus muscle followed by immunohistochemistry with an anti-ASIC1a antibody on DRG sections revealed that about 50% of neurons showed ASIC1a immunoreactivity (Hoheisel et al., 2004), and this is in line with the general assumption that acidosis is a mediator of muscle pain.

Conversely to *in situ* hybridization experiments where different probes can be easily synthesized and used to confirm the specifity of the obtained signal, immunohistochemical localization depends typically on the specificity of just one antibody used. Thus, immunohistochmical localizations should in our opinion always be considered with caution until the results are confirmed with different independent antibodies or otherwise verified (e.g., with knockout mice).

C. Native ASIC-Like Currents Characterized in Sensory Neurons

Petruska *et al.* (2002) characterized the chemosensitivity of mediumdiameter DRG sensory neurons. They detected amiloride-sensitive ASIClike currents in DRG neurons that expressed CGRP, substance P, or both peptides. A different study suggested that ASICs and TRPV1 are the major acid-sensitive ion channels in small-diameter DRG neurons (Liu *et al.*, 2004). Nineteen percent of IB4 positive and 10% IB4 negative small-diameter neurons responded to extracellular acid only with amiloride sensitive transient ASIC-like currents. Twenty-seven percent of IB4 positive and 69% of IB4 negative neurons had both ASIC-like and capsazepine sensitive acid activated currents. The group of Michael Welsh (Benson et al., 2002; Xie et al., 2002) compared the properties of heterologously expressed ASICs with those of H⁺-gated cation channels in medium to large sensory neurons from wild type and ASIC1, ASIC2, and ASIC3 null mice. A knockout of either ASIC1, ASIC2, or ASIC3 caused distinct changes in the properties of H⁺-gated currents in sensory neurons. They suggested that the ASICs in sensory neurons are mainly heteromultimers of two or three different subunits and can be mimicked by coexpression of ASIC1a, ASIC2a, and ASIC3. However, there are subpopulations of DRG sensory neurons that have only homomultimeric ASIC1a currents, blocked by the ASIC1a specific toxin PcTX1 (Escoubas et al., 2000). Thus, as expected, the ASICs in sensory neurons are heterogenous. Therefore, the characterization of native ASICs in subpopulations of sensory neurons that inervate a tissue where a role of ASICs in nociception is suspected will be increasingly important for the understanding of the physiological role of ASICs. Acidosis accompanies cardiac ischemia and is a suspected mediator of angina pain (Jacobus et al., 1977). The group of McCleskey (Benson et al., 1999; Sutherland et al., 2001) labeled sensory neurons that inervate the heart by injection of a retrogradely transported fluorescent dye (DiI®) into the pericardial space and characterized the H⁺-gated currents in labeled neurons in primary cultures. They recorded rather huge H⁺-gated cation currents (average 13 nA) with a pH dependence, kinetics, and a block by Ca^{2+} closely resembling those of the heterologously expressed ASIC3 subunit. Furthermore, cardiac sensory neurons have only very small TRPV1 currents suggesting that ASIC3 is the predominant acid sensor there. Acidosis is also candidate for mediating muscle pain. Using retrograde labeling of muscle innervating sensory neurons, Sluka et al. (2003) showed that (1) about 50% of 19-39 µm diameter sensory neurons responded to a pH 5 stimulus with an inward current and (2) a majority (6 out of 10) of acid sensitive sensory neurons examined responded with a very rapidly inactivating current that was followed by a sustained current in half of them.

VII. ROLE OF ASICs IN NOCICEPTION

A. Do ASICs Meet the Requirements for an Acid Sensor in Pain Perception?

Pain is induced when the tissue pH drops below neutral. ASIC1a, ASIC1b, and the transient ASIC3 current are highly acid sensitive and have

dose-response curves well suited to detect physiologically relevant acidosis (Waldmann and Lazdunski, 1998; Sutherland *et al.*, 2001).

ASIC1a and ASIC1b inactivate almost completely within seconds (τ ASIC1a = 3.5 s, τ ASIC1b =1.7 s) and require a rather long return to neutral pH to recover from inactivation (τ ASIC1a = 13 s, τ ASIC1b = 5.9 s) (Sutherland *et al.*, 2001). Thus, ASIC1a and ASIC1b could sense the onset of an acidosis, but sustained acidosis would inactivate both ASIC1 splice variants. The low-pH sensitivity of ASIC2a (pH_{0.5} = 4.5) does not suggest that acid activation of homomultimeric ASIC2a channels plays an important role in nociception.

The transient ASIC3 current is highly acid sensitive (Waldmann *et al.*, 1997a; de Weille *et al.*, 1998) and can be activated when the pH drops just slightly below pH 7.4 (Sutherland *et al.*, 2001) in the presence of low-extracellular Ca²⁺ concentrations (e.g., Ca²⁺ complexation by lactate). However, ASIC3 desensitizes very rapidly ($\tau = 0.32$ s) (Sutherland *et al.*, 2001) and requires an increase of the extracellular pH for recovery. Thus, the transient ASIC1 and ASIC3 currents appear to be well suited to sense pH fluctuations.

ASIC3 is the principal ASIC in cardiac sensory neurons (Sutherland *et al.*, 2001) and seems to be also expressed in sensory neurons innervating skeletal muscle (Sluka *et al.*, 2003). While the global pH in most tissues only changes slowly, local pH will probably be less stable. Under physiological conditions, bicarbonate is a very efficient buffer to correct slow pH fluctuations. However, especially in the absence of carbonic anhydrase bicarbonate buffer is rather slow. One should keep in mind that pH 7.4 means just 24 protons per μ m³, pH 7.2 corresponds to 38 protons per μ m³. Contracting muscle has a rhythmically fluctuating metabolic activity and might generate local pH fluctuations that are sufficiently important to activate highly acid sensitive ASICs, such as ASIC3, when the global tissue pH drops slightly below neutral and when lactate accumulates in the extracellular space. ASIC3 recovers rapidly from inactivation (Table I) and could respond to very small-repetitive pH fluctuations. Thus, sensing pH fluctuations might be the principle function of ASIC3 in skeletal or cardiac muscle.

ASIC3 has a sustained component activated at very low pH (<pH 5). Sensing such extreme free proton concentrations might be of physiological importance in the gastrointestinal tract (Holzer, 2003). The presence of ASIC3 immunoreactivity in the intestine also suggests a possible role of ASIC3 there (Yiangou *et al.*, 2001).

During many painful pathologies that are associated with a tissue acidosis (e.g., inflammation), the tissue pH is thought to decline slowly and remain acidic for hours or days. Such a sustained acidosis would lead to a profound inactivation of the highly acid sensitive ASICs, ASIC1a and ASIC3. How

could ASICs increase activity of sensory nerve endings under sustained acidic pH?

i. For several ASICs, a small sustained activity was reported during prolonged application of acid (Deval *et al.*, 2003). Sensory neurons, such as those innervating the heart, express huge ASIC currents, and a just very partial activation might suffice to cause firing of sensory neurons (Benson *et al.*, 1999).

ii. Coactivators, such as neuropeptides (e.g., neuropeptides FF), alter desensitization kinetics and might play a role in modulating ASIC mediated pain (Askwith *et al.*, 2000; Catarsi *et al.*, 2001; Deval *et al.*, 2003) during prolonged acidosis.

iii. Of course, as for yet unknown post translational modifications or associated proteins might completely alter the kinetics of ASICs.

B. Evidence for a Role of ASICs in Acid-Induced Pain Perception and Hyperalgesia

Initial studies using intracutaneous perfusion in human volunteers suggested that acid induces long lasting, nonadapting pain (Steen and Reeh, 1993; Steen et al., 1996). Since severe inflammation, ischemia, cancer, or arthritis is associated with pronounced and long lasting tissue acidosis, those data suggested that acid is an important mediator of the pain associated with those pathologies. Conversely, a study using noninvasive transdermal iontophoresis of H⁺ showed that acid-induced cutaneous pain was transient (Jones et al., 2004). Pain was induced within seconds and returned close to the baseline within 5 min during extended acid iontophoresis. Furthermore, the skin area remained almost completely desensitized toward acid stimuli during the first hour following a 4 min acid iontophoresis, and complete recovery of the acid sensitivity took 24 hrs. The study also showed that acidinduced pain was partially inhibited by NSAIDs, known blockers of ASIC1 and ASIC3. Conversely, pain sensation of the volunteers were unaffected by capsazepine, a competitive capsaicin antagonist at TRPV1 channels. In conclusion, the authors suggested that ASICs and not TRPV1 were the principle mediators of this transient acid-induced pain.

ASICs are expressed in sensory neurons, and application of acid to primary cultures of sensory neurons provokes transient action potential discharge. The transient pain perceived by the human volunteers in the study by Jones *et al.* (2004) is probably partially caused by such desensitizing ASIC activation. However, the very transient activation and profound desensitization of the pain responses sheds doubts on the relevance of such ASIC mediated pain responses (4 min pain, hours of insensibility) for painful pathologies, such as inflammation, associated with long lasting acidosis. Conversely, ASIC mediated desensitizing pain responses appear well suited to induce amplified pain responses during the onset of an ischemia.

Two groups reported an altered acid mediated pain in ASIC3 null mice. Chen et al. (2002) surprisingly observed increased pain reponses in the acetic acid (0.6% i.p.) induced writhing test with ASIC3 null mice. At lower acid concentration (<0.4%), there was no difference between both genotypes. Unlike wild-type mice, ASIC3 null mice had a very pronounced vocal pain response during the i.p. acid injection (Chen et al., 2002). In large-diameter DRG sensory neurons of ASIC3 null mice, a slower inactivation at pH 6 $(ASIC3 - /-: \tau = 2.60 \pm 0.40 \text{ s}; \text{ wild-type: } \tau = 0.15 \pm 0.01 \text{ s})$ (Benson *et al.*, 2002) and a slightly increased amplitude of ASIC-like currents stimulated at pH 5 was reported (Xie et al., 2002). Due to the much slower desensitization, the acid stimulated total charge flow into those sensory neurons would be higher for ASIC3 null mice. Those differences in kinetics may be the reason for the increased transient pain response of ASIC3 null mice after i.p. acid injection. Heterologous coexpression of ASIC subunits revealed the likely molecular basis for those findings (Benson et al., 2002). Inactivation of ASIC1a ($\tau = 4.0 \pm 0.35$ s) was much slower than of ASIC3 ($\tau = 0.32 \pm$ 0.02 s). When both subunits were coexpressed, the heteromultimeric channel inactivated faster than either homomeric channel ($\tau = 0.14 \pm 0.01$ s) and with similar kinetics as the native ASIC-like current ($\tau = 0.15 \pm 0.01$ s) in a population of large-diameter sensory neurons. Recovery from inactivation was much slower for homomultimeric ASIC1 channels than for heteromultimeric ASIC1a/ASIC3 channel ($\tau = 11$ s versus 0.64 s) (Benson *et al.*, 2002). Thus, the expected effect of an ASIC3 knockout on sensory neurons expressing ASIC1a/ASIC3 heteromultimeric channels would be an increased response to single strong acid stimuli followed by a longer period of inactivity. However, when injected into the paw, 0.6% acetic acid provoked similar licking responses in wild type and ASIC3 null mice in another study (Price et al., 2001). This might be possibly due to a different ASIC subunit combination expressed in cutaneous sensory neurons as compared to nociceptive afferents signaling abdominal pain after acid injection. A difference in the actual time course (injection speed) or magnitude of the pH change after injection of 20 μ l acetic acid into the paw or that of 200–300 μ l (10 μ l/g) i.p. might also be an issue.

Price *et al.* (2001) reported that ASIC3 null mutant mice developed a less pronounced mechanical hyperalgesia 4 h after intramuscular injection of unbuffered pH 4 saline than wild-type mice. The authors confirmed and refined this study later (Sluka *et al.*, 2003). Bilateral mechanical hyperalgesia developed in wild-type mice after two injections of pH 4 saline 5 days apart,

and it lasted for 4 weeks. Conversely, ASIC3 knockout mice did not develop mechanical hyperalgesia, suggesting that activation of homo- or heteromultimeric ASIC3 channels triggers hyperalgesia in this experimental setup. Somewhat puzzling is the question how such a low concentration (100 μ M) of unbuffered acid injected into the strongly buffered environment of a living tissue can cause sufficient activity of ASIC3 and lead to long lasting changes. The acidosis after intramuscular pH 4 saline injection can only be very transient. Thus, in that model of muscular hyperalgesia, two very brief activations of ASIC3 channels in muscular afferents were sufficient to induce lasting hyperalgesia. ASIC3 was potentiated at low-Ca²⁺ concentrations (Immke and McCleskey, 2003), and the injection of Ca^{2+} free acidic solutions in the study by Sluka et al. (2003) might have potentiated the effect of acid. Conversely to the ASIC3 knockout, disruption of the ASIC1 gene did not affect mechanical hyperalgesia after intramuscular acid injection despite the fact that ASIC1a protein was detected in muscle inervating sensory neurons (Sluka et al., 2003; Hoheisel et al., 2004).

The data obtained from ASIC3 null mice show that this ion channel subunit plays a role in pain perception and hyperalgesia in the experimental setups used. However, i.p. injection (10 ml/kg) of acetic acid or intramuscular injection of acid is treatment that is fortunately not frequently experienced in real life. It will be interesting to see whether a metabolic muscle acidosis also leads to altered pain responses with ASIC3 knockout mice. Inflammation is usually associated with tissue acidosis. However, until now pain behavior in models for inflammatory pain (e.g., after carrageenan injection) was unaltered in ASIC3 null mice (Price *et al.*, 2001; Chen *et al.*, 2002).

Despite the expression of ASIC1 in the peripheral nervous system and the high-acid sensitivity of this channel, no altered acid mediated nociception after a knockout or block of ASIC1 was reported as for yet.

C. ASICs and Mechanosensation

Protons and fluctuations of the extracellular Ca^{2+} concentration (for ASIC3) are currently the only known activators of ASICs. It is possible that others as for yet unknown activators (e.g., peptides) are the physiological relevant ones. Stretch activation of ASICs was proposed but never demonstrated in an *in vitro* expression system. ASICs are homologs of the degenerins of the nematode *C. elegans* (Huang and Chalfie, 1994; Ernstrom and Chalfie, 2002). Certain degenerins (MEC-4, MEC-10) are required for body touch sensation of the nematode suggesting that they might be subunits of a stretch activated cation channel (Huang and Chalfie, 1994;

Ernstrom and Chalfie, 2002). During the past years indirect evidence for this hypothesis was accumulating and recent data virtually proved it. The group of Miriam B Goodman recorded mechanosensitive currents from C. elegans touch receptors in vivo and showed that MEC-4 and MEC-10 mutations associated with lateral body touch insensitivity of the nematode caused loss of the mechano-stimulated cation currents (O'Hagan et al., 2005). The final proof would be a reconstitution of the C. elegans touch transducer channel in a heterologous expression system or lipid bilayers, which might turn out difficult to achieve since numerous associated proteins apparently seem to be required for a correct function of the degenerin mechanosensitive channel (Ernstrom and Chalfie, 2002). Certain forms of mammalian mechanosensation, such as hearing, were affected by amiloride, a blocker of ASIC and other channels. Thus, ASICs appeared likely candidates for mammalian mechanosensitive cation channels. It was not surprising that the first published phenotype of ASIC null mice was an impaired mechanosensation (Price et al., 2000). However, the effect of ASIC inactivation turned out much smaller than initially anticipated, and there is currently increasing evidence that ASICs do not play an important role in mechanosensation. The initially reported principal touch sensation phenotype of ASIC2 null mutant mice was a decreased stimulus-response coding of rapidly activating mechanosensitive fibers in the nerve-skin model (Price et al., 2000). However, the mechanical von Frey thresholds were unaltered, which was a rather unexpected finding if the animals lacked one major constitutent of the molecular "mechanosensor" complex.

ASIC3 null mice displayed a somewhat increased stimulus-response coding of rapidly activating fibers (Price et al., 2001). Again the von Frey thresholds of RA fibers were unaltered in ASIC3 null mice. An increased sensitivity toward rather strong mechanical stimuli (tail pressure) of ASIC3 null mutant mice was reported by the group of Zimmer (Chen et al., 2002). By analogy with the multisubunit model proposed for the C. elegans mechanosensitive ion channel, Price et al. (2001) proposed that ASIC3 was also part of a multisubunit mechanosensative cation channel and that loss of ASIC3 rendered the channel more mechanosensitive. Unaltered cutaneous mechanosensation and a somewhat increased mechanosensitivity of colonic and gastroesophagal mechanosensitive fibers was reported with ASIC1 knockout mice (Page et al., 2004). The role of ASICs in mechanosensation became more controversial. The group of J. Wood studied mechanosensitive cation currents in primary cultures of sensory neurons from wild type and ASIC2 or ASIC3 null mutant mice (Drew et al., 2004). They classified different neuronal subpopulations depending on soma size, isolectin B4 (IB4) binding, and action potential duration. Mechanosensitive cation currents were highest in the neuronal subpopulation thought to be low

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threshold mechanosensitive *in vivo* (Drew *et al.*, 2002). However, currents were unaltered in sensory neurons from ASIC2 or ASIC3 null mice, a finding that strongly argues against an important role of ASIC2 or ASIC3 in mechanosensation (Drew *et al.*, 2004). Since mechanosensation was a suspected role of ASICs, we also studied mechanosensation in ASIC2 null mice (Roza *et al.*, 2004). Conversely to a previous study (Price *et al.*, 2000), we did not find a decreased cutaneous mechanosensation in ASIC2 null mice. Furthermore hearing and visceral mechanonociception of the colon (CGRP release) were unaltered in our study. Taken together, the data rather suggest that pathways independent of ASIC activity are predominant in mammalian mechanosensation.

D. ASICs in Spinal Cord

ASIC transcripts for ASIC1a, ASIC2a, and ASIC2b have been found at a high level in dorsal horn neurons (Wu *et al.*, 2004). Electrophysiological and pharmacological properties of the currents in the majority of dorsal horn neurons were similar to those of homomeric ASIC1a and/or heteromeric ASIC1a + 2b channels. Peripheral complete Freund's adjuvant-induced inflammation resulted in increased expression of both ASIC1a and ASIC2a in dorsal horn. These results support the idea that ASICs may participate in central sensory transmission/modulation under physiological conditions and may play important roles in inflammation-related persistent pain (Wu *et al.*, 2004).

VIII. CONCLUSIONS

Since the cloning of the ASICs, a wealth of data regarding biophysical properties, modulators, and expression patterns of ASICs accumulated. Some findings point toward a physiological role of ASICs as acid sensors involved in nociception. Conversely, a crucial role of ASICs in mechanosensation appears currently unlikely.

A. ASICs and Mechanosensation

Members of the ENaC/Deg family of ion channels are not the only candidates for mechanosensitive cation channels. Loss of degenerin functions caused impaired lateral body touch sensation in *C. elegans*. Conversely, nose touch sensation remained intact and required the TRP channel OSM-9

(Colbert et al., 1997). Another TRP channel NompC was essential for mechanoreceptor potentials in both Drosophila tactile bristles (Walker et al., 2000) and Zebrafish sensory hair cells of the inner ear (Sidi et al., 2003). Data suggested a TRP channel as part of the mammalian cochlear hair cell transduction channel (Corey et al., 2004). Thus, evidence points toward a role of TRP channels in mechanosensation in several metazoan phyla. ENaC/Deg channels apparently form mechanosensitive cation channels in the nematode (O'Hagan et al., 2005), and there are some indications that ENaC/Deg channels might be involved in spider mechanosensation. Mechanosensitive channels of the slit-sense organ of spiders are Na⁺ selective, blocked by amiloride and potentiated by extracellular acid (Hoger and French, 2002), typical properties of ENaC/Deg channels. However, despite the plenty of ENaC/Deg channel genes in Drosophila (Fig. 2), no altered mechanosensation was associated with loss of ENaC/Deg channel function. The different phyla studied are separated by 500 million to one billion years of evolution. During evolution of modern nematodes, insects, and mammals from a common ancestor, different potential mechanosensitive cation channels were probably selected among the TRP, ENaC/Deg, and maybe other ion channel families. While it is unlikely that ASICs play an important role in cutaneous mechanosensation and hearing, it can of course not be ruled out that certain ASIC subunit combinations can form mechanosensitive channels that play a role in very specific forms of mechanosensation or just in certain subtypes of sensory neurons, which remains to be discovered.

B. ASICs and Acid Sensing

ASICs with varying subunit combinations are present in roughly half of the sensory neurons examined. Both knockout of ASIC genes (Benson *et al.*, 2002) or pharmacological block of ASICs (Escoubas *et al.*, 2000; Diochot *et al.*, 2004) resulted in impaired H⁺-gated inward currents in sensory neurons. Thus, ASICs appear an important component of the acid response mechanism of sensory neurons. However, the acid mediated nociception phenotypes of the ASIC knockout mice clearly did not meet the expectations. No such phenotype was described for ASIC1 and ASIC2 null mice. The ASIC3 knockout caused altered pain responses after acid injection. While those data demonstrate that activation of ASIC3 channels can cause pronounced changes in pain perception, the demonstration that a metabolic acidosis (e.g., after exercise) or an acidosis caused by inflammation is associated with altered pain responses in ASIC null mice is still lacking.
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Pain associated with ischemia or inflammation lasts until the end of the pathological condition. Thus, considerable research efforts focused on coactivators that transform the rapidly desensitizing ASIC currents into sustained currents. A small sustained activity at slightly acidic pH was reported for ASIC1 and ASIC3 and might be sufficient to cause nociceptor firing in sensory neurons that express high levels of ASIC1 or ASIC3. Neuropeptides and perhaps other as for yet unknown substances slow down ASIC1 and ASIC3 desensitization and might cause longer lasting nociceptor activation. In tissues with fluctuating metabolic activity (e.g., cardiac or skeletal muscle), local pH fluctuation might be the physiological relevant activator of ASICs that recover rapidly from inactivation such as ASIC3. Modulating or inducing nociceptor firing during lasting pain by repetetive or sustained ASIC activity might not even be the predominant role of ASICs in nociception. Just two brief activations of ASIC3 were sufficient to induce lasting mechanical hyperalgesia (Section VII.B.) (Sluka et al., 2003). Thus, the transient responses of ASICs during the onset of an acidosis can have pronounced long lasting effects on nociception.

C. Multiple Pathways for Acid and Mechanosensing Might Coexist

While completely redundant proteins are lost during evolution, population genetics also predicts that proteins with highly overlapping functions are usually maintained if they add only a very tiny advantage to an organism.

Thus, different pathways to sense acid or mechanical stress might coexist in mechanosensing or acid sensing sensory neurons, respectively. Such redundant pathways are the likely cause of the surprisingly weak nociception phenotypes of mice with targeted deletions of ion channels that appeared well suited to sense noxious signals (ASICs, TRPV1).

ENaC/Deg and TRP channels initially apeared the most appealing candidates for metazoan mechanosensors. However, indirect mechanisms of mechanosensing such as mechano-stimulated release of messenger molecules such as ATP and autocrine or paracrine activation of the respective receptors might also contribute to mechanosensing (Wynn *et al.*, 2003).

Acid activation of ASICs or TRPV1 and acid block of background K^+ channels (Chapter 15) probably all contribute to the acid-induced firing of sensory neurons. Apart from ion channels, most biochemical pathways are pH dependent, resulting in a multitude of potential acid sensing mechanisms that might also contribute to acid mediated nociception *in vivo*. The ASIC null mice so far did not provide the proof of a physiological function of the

ASICs but some hints that acid activation of ASICs has an important potential to modulate nociception *in vivo*.

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CHAPTER 9

P2X Receptors in Sensory Neurons

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I. OVERVIEW

P2X receptors are a family of ligand-gated ion-channels activated by ATP. Seven subtypes of P2X receptors have been identified. A unique distribution of P2X₃ subunits to nociceptors gave us much attention to their roles in the pain sensation. Efforts focusing on the distribution and electrophysiological properties of P2X receptor in sensory neurons revealed that homomeric P2X₃ receptors and heteromeric P2X_{2/3} receptors might be predominant functional P2X receptors in sensory neurons and play roles in generating discharges in the periphery and in modulating synaptic transmission in the dorsal horn. Together with behavioral evidence, P2X₃ and/or P2X_{2/3} receptors might detect ATP released from damaged tissues at periphery sensory endings, thereby inducing or modulating pain. However, neither blocking P2X receptors pharmacologically nor suppressing their expression molecularly and genetically affected basal responses to physiological pain (e.g., noxious heat or mechanical stimuli). Investigations have been more focused on roles of ATP signaling via P2X receptors in the pathogenesis of chronic pain including persistent inflammatory pain and neuropathic pain. Chronic pain states accompany alterations of several P2X receptors in sensory neurons. Behavioral studies using antagonists, antisense, or short-interference RNA specific targeting P2X₃ have shown that P2X₃ and/or P2X_{2/3} receptors at either peripheral or central terminals have an ongoing role as well as developmental role in pathological pain. Further investigations to determine how ATP signaling via P2X receptors participates in the pathogenesis of chronic pain will lead us closer to understanding the molecular mechanisms of pathological pain and be able to provide clues to developing new therapeutic drugs.

II. INTRODUCTION

Extracellular ATP may be an important substrate in the formation of pain (Burnstock, 1996). The first clue to this possibility was found about 30 years ago in clinical studies showing that ATP applied to blister bases (Bleehen et al., 1976; Bleehen and Keele, 1977) or injected intradermally (Coutts et al., 1981) was able to induce a pain sensation in humans. Electrophysiological studies further demonstrated that ATP can activate primary sensory neurons, suggesting the existence of receptors that detect ATP. Significant advances in our understanding of the molecular mechanisms of ATPinduced pain were made by the discovery of cell-surface receptors that detect extracellular ATP and other nucleotides, namely P2 receptors, on sensory neurons. Like other neurotransmitter receptors, P2 receptors are also classified into two families: ligand-gated ion-channel receptors, namely P2X receptors, and G-protein-coupled receptors, namely P2Y receptors (Ralevic and Burnstock, 1998; Khakh et al., 2001; North, 2002). Among the seven known subunits in the P2X family $(P2X_1 - P2X_7)$, the expression of $P2X_3$ was highly restricted to nociceptors (Chen et al., 1995). This discovery prompted us to focus on both ATP and P2X receptors as pain molecules and to develop useful tools for investigating their physiological roles. Combined with in situ hybridization studies, selective antibodies to each P2X receptor subtype revealed the expression profile of P2X receptors in primary sensory neurons as well as other regions in the body. Furthermore, using selective

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pharmacological (e.g., agonists and antagonists), molecular (e.g., antisense oligonucleotides and short-interference RNAs [siRNAs]), and genetic tools (e.g., gene-knockout animals) it began to appear that ATP released from damaged cells causes a pain sensation by activating P2X receptors on sensory neurons, which might serve as a defense mechanism by warning us of existing or imminent damage to the body. Moreover, evidence has also suggested that P2X receptors on sensory neurons contribute to the pathogenesis of chronic pain including inflammatory pain and neuropathic pain, the latter of which is intractable pain that does not resolve even though the overt tissue damage has healed (Woolf and Salter, 2000; Scholz and Woolf, 2002). In this article, we focus on developments in the understanding of the mechanisms by which P2X receptors on sensory neurons participate in the pathogenesis of chronic pain, as well as summarize the accumulated evidence from immunohistochemical, electrophysiological, and behavioral studies, and we provide a framework for addressing the major questions that remain.

III. MOLECULAR STRUCTURE AND PHARMACOLOGICAL PROFILES OF P2X RECEPTORS

P2X receptors belong to a family of ligand-gated ion-channels and are cation-selective channels with almost equal permeability to Na⁺ and K⁺ and significant permeability to Ca²⁺ (Ralevic and Burnstock, 1998; Khakh *et al.*, 2001; North, 2002). Molecular cloning has so far identified seven genes encoding P2X receptor subunits (P2X₁–P2X₇). All P2X subunits possess two transmembrane regions and have intracellular N- and C-termini and a long extracellular loop between the transmembrane domains (Khakh *et al.*, 2001; North, 2002; Vial *et al.*, 2004). One third of the amino acids in the extracellular loop are conserved in at least six P2X subunits, suggesting their involvement in ATP binding (Vial *et al.*, 2004). While the intracellular N-terminal regions have relatively similar lengths of amino acids, the length of the C-termini diverges considerably from 30 residues in P2X₆ to 240 in P2X₇ (North, 2002; Vial *et al.*, 2004). In addition, there is a putative motif for phosphorylation by protein kinase C and A in the N- and C-termini, respectively (North, 2002).

One functional P2X receptor channel is presumably formed by a number of P2X subunits, as is considered to be the case in other ligand-gated ion channels. The number of subunits of a P2X receptor has been proposed to be three (Nicke *et al.*, 1998; Stoop *et al.*, 1999; Jiang *et al.*, 2003; Nicke *et al.*, 2003) or four (Kim *et al.*, 1997; Ding and Sachs, 2000). It appears that all three or four subunits are either identical, namely homomeric receptors, or not identical, namely heteromeric receptors. Lewis *et al.* (1995) first showed the ability of P2X₃ to form heteromeric receptors with P2X₂ (P2X_{2/3}). Biochemical approaches using co-immunoprecipitation have shown that all P2X subunits (excepting P2X₇) can heteropolymerize with any of the other P2X subunits (Radford *et al.*, 1997; Torres *et al.*, 1999). The co-assembly of P2X_{1/5} (Torres *et al.*, 1998), P2X_{2/3} (Lewis *et al.*, 1995), P2X_{2/6} (King *et al.*, 2000), or P2X_{4/6} (Le *et al.*, 1998a) heteromeric receptors has been demonstrated to form functional receptors as well. In addition, there is a report that the P2X_{2/3} receptor seems likely to contain one P2X₂ and two P2X₃ subunits (Jiang *et al.*, 2003).

Each of the seven homomeric P2X receptors and at least four heteromeric receptors shows different (but partly overlapping) electrophysiological and pharmacological properties in terms of current kinetics, desensitization rates, and sensitivities to agonists and antagonists (Ralevic and Burnstock, 1998; Khakh et al., 2001; North, 2002). When each one or two of them are expressed heterologously in cells, ATP evokes a rapid- or slow-inactivating inward current. The former is seen in cells expressing either the $P2X_1$ or $P2X_3$ receptor, and the latter is seen in those expressing all other receptors including the four heteromeric receptors (Khakh et al., 2001; North, 2002). Furthermore, repetitive activation of $P2X_1$ and $P2X_3$ receptors by ATP shows a marked desensitization of the ATP-induced responses. α,β -methylene ATP ($\alpha\beta$ meATP), an analog of ATP, is a useful agonist for basically identifying P2X receptors containing P2X₁ or P2X₃ subunit (i.e., P2X₁, P2X₃, P2X_{1/5}, and P2X_{2/3}), although $\alpha\beta$ meATP also activates P2X_{4/6} (Ralevic and Burnstock, 1998; Khakh et al., 2001; North, 2002) (Table I). As for antagonists, suramin and pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) have an antagonistic property to almost all P2X receptors except the rat P2X₄ receptor (Buell et al., 1996). However, it should be noted that suramin also blocks several neurotransmitter receptors (e.g., NMDA and GA-BAA receptors) (Nakazawa et al., 1995; Peoples and Li, 1998). 2',3'-O-(2,4,6trinitrophenyl)-ATP (TNP-ATP) at a nanomolar range selectively blocks P2X1, $P2X_{3}$, and $P2X_{2/3}$ receptors (Virginio *et al.*, 1998) and thus would be a useful tool to distinguish those from the other P2X receptors that are about 1000 times less sensitive to TNP-ATP (Virginio et al., 1998). Furthermore, diinosine pentaphosphate (Ip₅I) is a very potent antagonist to P2X₁ (King *et al.*, 1999) and P2X₃ receptors but not to $P2X_2$ and $P2X_{2/3}$ receptors (Dunn *et al.*, 2000). This is the only antagonist for distinguishing between P2X₃ and P2X_{2/3}. Jarvis et al. (2002) developed the first selective antagonist for P2X₃ (and P2X_{2/3})—A-317491. A-317491 blocks the responses mediated by P2X₃ or P2X_{2/3} receptors in a competitive fashion without any effect on other receptors, enzymes, and ion-channels.

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	Agonist (EC50)				Antagonist (IC50)		
	ATP	$\alpha\beta$ meATP	Suramin	PPADS	TNP-ATP	Ip ₅ I	A-317491 ^c
P2X ₁	1	1–3	1	1	0.006	0.003	>10(12)
P2X ₂	10	>300	10	1	1	Inactive	>10(47)
P2X ₃	1	1	3	1	0.001	3	0.1
$P2X_4$	10	>300	>300	>500	15	Potentiation	>100
P2X ₅	10	>300	4	>500	0.45^{b}	$>30^{c}$	
P2X ₇	100	>300	~ 500	3	>30	>100	
P2X _{1/5}	1	3	1.6	0.6	0.4		
P2X _{2/3}	1	1–3	30–100	3-300	0.007	Inactive	0.17
P2X _{2/6}	30	>100	6				
P2X _{4/6}	10	30	10	10	$>1^a$		

 TABLE I

 Pharmacological Properties of Recombinant Homomeric and Heteromeric P2X Receptors

^aTsuzuki et al. (2003).

^bWildman et al. (2002).

^cJarvis et al. (2002).

For more details, see North, R. A., and Surprenant, A. (2000). Annu. Rev. Pharmacol. Toxicol. 40, 563–580, Khakh et al. (2001), and Dunn et al. (2001).

IV. ELECTROPHYSIOLOGICAL RESPONSES FOLLOWING THE ACTIVATION OF P2X RECEPTORS IN SENSORY NEURONS

Electrophysiological studies using acutely dissociated and cultured primary sensory neurons from dorsal root ganglia (DRG), trigeminal ganglia (TG), and nodose ganglia (NG) have shown that functional P2X receptors are expressed. The application of ATP extracellularly evokes an inward current (Krishtal et al., 1983, 1988; Bean, 1990; Bouvier et al., 1991; Khakh et al., 1995; Lewis et al., 1995; Robertson et al., 1996; Cook et al., 1997; Gu and MacDermott, 1997; Rae et al., 1998; Burgard et al., 1999; Grubb and Evans, 1999; Ueno et al., 1999; Labrakakis et al., 2000; Petruska et al., 2000; Tsuda et al., 2000; Cook and McCleskey, 2002; Tsuzuki et al., 2003), intracellular Ca2+ transient (Bouvier et al., 1991; Tsuda et al., 1999a; Nakatsuka et al., 2001), depolarization (Jahr and Jessell, 1983), and action potentials (Cook et al., 1997; Cook and McCleskey, 2002). These responses are inhibited by antagonists for P2X such as suramin or PPADS (Robertson et al., 1996; Cook et al., 1997; Gu and MacDermott, 1997; Rae et al., 1998; Grubb and Evans, 1999). Depending on the study, the percentage of sensory neurons that respond to ATP varies between 40% and 96%, but this could be due to the age of the animals from



FIGURE 1 Two distinct types of electrophysiological responses in DRG neurons and pain behaviors evoked by activation of P2X receptors. The application of $\alpha\beta$ meATP (100 µM) to acute isolated DRG neurons evokes a transient inward current (A), left, or a persistent current (B), left. The transient ATP currents are predominantly seen in DRG neurons that respond to capsaicin (A), middle, and the persistent ones are mainly seen in capsaicin-insensitive DRG neurons (B), middle. Right graphs in (A) and (B) show the distribution (X-axis: size of neurons, Y-axis: amplitude) of DRG neurons that responded to $\alpha\beta$ meATP (100 µM) with transient (A) and persistent (B) currents and that were acutely isolated from normal rats or neonatal capsaicintreated rats. The DRG neurons that responded to $\alpha\beta$ meATP (100 µM) with transient current disappeared in neonatal capsaicin-treated rats (A), right, whereas that with persistent current remained (B), right. Intraplantar injection of $\alpha\beta$ meATP (100 nmol) causes nocifensive behavior (C) and mechanical allodynia (D). While the $\alpha\beta$ meATP-induced nocifensive behavior

which the DRG neurons were isolated because the expression of P2X receptors changes developmentally (Boldogkoi et al., 2002; Cheung and Burnstock, 2002). Furthermore, the ATP-evoked inward currents in sensory neurons show heterogeneity in terms of both the current kinetics and desensitization rates. Cook et al. (1997) have shown that the inward currents evoked by ATP or $\alpha\beta$ meATP in nociceptors from TG can be kinetically divided into two populations: one shows a transient inward current that is readily desensitized by repetitive application of agonists, the other shows a persistent and nondesensitizing current. The same heterogeneity is also seen in DRG neurons (Burgard et al., 1999; Grubb and Evans, 1999; Ueno et al., 1999; Labrakakis et al., 2000; Petruska et al., 2000; Tsuda et al., 2000; Tsuzuki et al., 2003) (Figs. 1A and B). The transient ATP currents are predominantly seen in DRG neurons that respond to capsaic (Fig. 1A), an activator of nociceptors (thereby causing burning pain). In contrast, the persistent ones are mainly seen in capsaicin-insensitive DRG neurons (Fig. 1B), implying that these types of responses may be classified by the presence of capsaicin sensitivity and that they have a distinct role in pain signaling (Ueno et al., 1999). Actually, these responses produce distinct phenotypes of pain (see later). In addition to the third type, which mixes both transient and persistent currents (Burgard et al., 1999; Grubb and Evans, 1999; Dunn et al., 2000; Labrakakis et al., 2000; Petruska et al., 2000; Liu et al., 2001; Tsuzuki et al., 2003), there seems to be at least three groups of neurons that respond to ATP in the DRG. The heterogeneity of the ATP responses suggests that they reflect the heterogeneity of the expression of P2X receptors in sensory neurons. Which of the seven P2X receptor subtypes are molecular targets for ATP to evoke inward currents in sensory neurons? As mentioned above, each of the seven P2X receptors has distinct pharmacological and electrophysiological profiles (Ralevic and Burnstock, 1998; Dunn et al., 2001; Khakh et al., 2001; North, 2002). In sensory neurons, transient, persistent, and mixed types, all are evoked by $\alpha\beta$ meATP as well as ATP (Robertson et al., 1996; Cook et al., 1997; Gu and MacDermott, 1997; Rae et al., 1998; Burgard et al., 1999; Grubb and Evans, 1999; Ueno et al., 1999; Labrakakis et al., 2000; Petruska et al., 2000; Tsuda et al., 2000; Cook and McCleskey, 2002; Tsuzuki *et al.*, 2003), but $\beta\gamma$ me-L-ATP, a potent agonist of the P2X₁ receptor, is less active (Rae et al., 1998), suggesting that the P2X receptor in sensory neurons contains P2X3 subunit(s). Furthermore, all types of ATP currents in sensory neurons are inhibited by TNP-ATP at a nanomolar range as well as by suramin and PPADS (Burgard et al., 1999; Grubb and Evans, 1999; Xu and Huang, 2002). Thus, the candidates might be P2X₃ and

disappeared in neonatal capsaicin-treated rats (C: ***p < 0.001 versus normal rats), mechanical allodynia was almost unchanged (D: *p < 0.05 versus normal rats). Reprinted from Tsuda *et al.* (2000).

 $P2X_{2/3}$. The ATP-evoked persistent and transient responses seen in sensory neurons are similar to those in cells in which recombinant $P2X_3$ is heterologously expressed with and without recombinant P2X₂, respectively (Lewis et al., 1995). Moreover, DRG neurons from mutant mice lacking P2X₃ do not show any transient currents evoked by ATP or $\alpha\beta$ meATP (Cockayne *et al.*, 2000; Souslova et al., 2000; Zhong et al., 2001). P2X₃ homomeric receptors must be the receptors for generating transient currents by ATP in sensory neurons. In contrast, ATP still produces persistent currents in P2X₃-deficient DRG neurons but $\alpha\beta$ meATP does not (Cockayne *et al.*, 2000; Souslova *et al.*, 2000; Zhong et al., 2001). Therefore, it might be that the persistent responses are mediated by activating $P2X_{2/3}$ receptors. Mutant mice lacking both $P2X_2$ and P2X₃ show few responses to ATP in their DRG neurons (Cockayne et al., 2005). In addition, the mixed type of ATP-evoked currents is predicted to be mediated by activating both P2X₃ and P2X_{2/3} simultaneously. The mixture of both currents in sensory neurons can be relatively mimicked in oocytes that express P2X₂ and P2X₃ subunits in different proportion (Liu et al., 2001), and have similar properties of P2X₃- and P2X_{2/3}-mediated ATP currents in terms of their sensitivities to agonists and antagonists, desensitization rates (Burgard et al., 1999; Grubb and Evans, 1999), and modulation by protons (Stoop et al., 1997; Burgard et al., 1999). Moreover, Ip₅I, an antagonist that can be used to distinguish between P2X3 and P2X2/3 receptors at a low concentration, inhibits only the transient, but not the persistent component in DRG neurons (Dunn et al., 2000). Additionally, there could be one more different type of persistent current in sensory neurons, which is evoked by $\alpha\beta$ meATP and is sensitive to PPADS, but not to TNP-ATP, suggesting $P2X_{4/6}$ and $P2X_{1/5}$ receptors (Tsuzuki et al., 2003). A very small persistent current in response to ATP in some DRG neurons from $P2X_2$ and $P2X_3$ double knockout mice has been observed (Cockayne et al., 2005). The presence of functional P2X receptors containing P2X subunits other than P2X₂ and P2X₃ is also indicated, but their expression levels might be low (Cockayne et al., 2005). Thus, under normal conditions, sensory neurons might essentially have three distinct types of ATP responses that could be mediated virtually by $P2X_3$, $P2X_{2/3}$, or both receptors.

V. DISTRIBUTION OF P2X RECEPTORS IN SENSORY NEURONS

In 1995, two groups first found that the mRNA of the P2X₃ receptor was selectively expressed at high levels in rat DRG and NG neurons (Chen *et al.*, 1995; Lewis *et al.*, 1995) and is immunohistochemically positive to peripherin, a marker of small sensory neurons (Chen *et al.*, 1995). Immunohistochemical studies utilizing antibodies specific to each P2X receptor strongly support the above findings. Immunoreactivity (ir) of P2X₃ receptors is found in

approximately 40% of all neurons in the DRG and TG that are predominantly small- to medium-sized (Cook *et al.*, 1997; Vulchanova *et al.*, 1997, 1998; Bradbury *et al.*, 1998; Eriksson *et al.*, 1998; Ramer *et al.*, 2001) and whose axons could project to the skin, viscera, and tooth pulp but not to the skeletal muscle (Cook *et al.*, 1997; Bradbury *et al.*, 1998). In the lumber DRG, P2X₃-ir is virtually not colocalized with NF200, an antineurofilament antibody that stains large-diameter neurons (Bradbury *et al.*, 1998). However, in more rostral DRG, such as the cervical DRG and TG, some of the large-sized neurons are actually positive to P2X₃-ir (Eriksson *et al.*, 1998; Ramer *et al.*, 2001), suggesting that the size distribution of P2X₃ in sensory neurons differs between rostral and caudal levels of the spinal segments.

The involvement of the P2X₃ receptor in pain signaling was strongly suggested by its colocalization with the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1), the expression of which occurs predominantly in nociceptors. P2X₃ mRNA and P2X₃-ir-positive DRG neurons are dramatically decreased in rats when sensory neurons that are sensitive to capsaicin have been destroyed by neonatal capsaicin treatment (Chen et al., 1995; Guo et al., 1999). This is substantially supported by the immunohistochemical evidence that the majority of P2X₃-ir-positive DRG neurons have TRPV1-ir (Vulchanova et al., 1998). Nociceptors are generally divided into two categories: one that expresses the nerve growth factor (NGF) receptor TrkA and requires NGF for survival, and the other that expresses the glial cell linederived neurotrophic factor (GDNF) receptor GFR α -1 and Ret and requires GDNF for survival (Averill et al., 1995; Molliver et al., 1997; Snider and McMahon, 1998). NGF-sensitive nociceptors contain neuropeptides, like substance P, while GDNF-sensitive nociceptors do not contain such peptides and are therefore called nonpeptidergic neurons (Averill et al., 1995; Molliver et al., 1997; Snider and McMahon, 1998). In cytochemical analyses of P2X₃positive DRG neurons using several markers of sensory neurons, almost all of the P2X₃ receptor-positive DRG neurons are labeled by isolectin B4 (IB4) and the enzyme fluoride-resistance acid phosphate (FRAP), both of which selectively label GDNF-sensitive sensory neurons, while few neurons are positive to substance P and somatostatin (Bradbury et al., 1998; Vulchanova et al., 1998). About 20% of P2X₃-ir DRG neurons, however, express calcitonin generelated peptide (CGRP) (Bradbury et al., 1998). These lines of evidence thus indicate that sensory neurons expressing P2X₃ belong predominantly, but not exclusively, to the group of GDNF-sensitive nonpeptidergic nociceptors.

The expression patterns of P2X₃ receptors in the mouse DRG and spinal cord are very similar to those in rats (Zhong *et al.*, 2001; Zwick *et al.*, 2002). However, a high proportion of rat DRG neurons express both P2X₃ and TRPV1 (Guo *et al.*, 1999), which appears not to be the case in the mouse DRG. While about 75% of P2X₃-ir mouse DRG neurons are positive to IB4,

surprisingly IB4-positive mouse DRG neurons virtually do not express TRPV1 (Zwick *et al.*, 2002). This may allow us to deduce that the proportion of P2X₃-positive neurons in which TRPV1 is coexpressed could be much lower in mice (<25%) than in rats (about 75%) (Guo *et al.*, 1999). In monkey, P2X₃-ir is seen in DRG neurons but not in the spinal cord (Vulchanova *et al.*, 1997). P2X₃ receptors are also found not only in small- and medium-sized human DRG neurons that are predominantly positive to IB4 (Yiangou *et al.*, 2000), but also in other tissue (mRNA) (Garcia-Guzman *et al.*, 1997). Accordingly, it is thus possible that the pain phenotype resulting from the activation of P2X₃ receptors may differ among species.

P2X₃ receptor stands out conspicuously from other P2X receptors both in terms of their expression levels and their restricted localization, but the existence of both mRNAs and the proteins of the other five P2X receptors (P2X₁, P2X₂, P2X₄, P2X₅, and P2X₆) are also found in sensory neurons (Collo et al., 1996; Xiang et al., 1998). P2X₂ receptor mRNA or its protein is detected in DRG neurons (Simon et al., 1997) of various sizes (Vulchanova et al., 1997) or are found predominantly in Ret-positive small-diameter DRG neurons, presumably nonpeptidergic nociceptors (Kobayashi et al., 2005). In addition, $P2X_2$ -ir is also observed in satellite glia (Vulchanova *et al.*, 1997). $P2X_2$ is often observed in P2X₃-positive neurons (Vulchanova et al., 1997; Bo et al., 1999; Kobayashi et al., 2005). This is consistent with electrophysiological data showing $P2X_{2/3}$ -like responses in sensory neurons (see the preceding section) and provides evidence for the existence of $P2X_{2/3}$ receptors in native sensory neurons. P2X4 mRNA is detected in almost all sizes of DRG neurons and in satellite glia (Kobayashi et al., 2005). P2X5 and P2X6 could be expressed preferentially in NF-200-positive large DRG neurons, likely myelinated A fibers (Kobayashi *et al.*, 2005). The expressions of $P2X_1$ and $P2X_7$ receptors occur quite rarely, if at all, in the DRG (Ueno et al., 2002; Kim et al., 2003; Kobayashi et al., 2005). In addition, colocalization of P2X₃ and P2X₅ is also found in 13% of all DRG neurons (Kobayashi et al., 2005). P2X4, P2X5, and P2X₆ mRNA all appear to be coexpressed in over 30% of all DRG neurons, which are virtually all TrkC-positive neurons considered to be a subpopulation of proprioceptors (Kobayashi et al., 2005).

VI. P2X RECEPTORS AT CENTRAL AND PERIPHERAL TERMINALS

As is generally considered to be the case with other proteins, P2X receptor proteins could be synthesized in the cell bodies of sensory neurons and then delivered to their nerve terminals in both the periphery and the dorsal horn of the spinal cord.

In the dorsal horn of the spinal cord, immunoreactivities to $P2X_1$, $P2X_2$, $P2X_3$, and $P2X_4$ are all seen mainly in the superficial layer (Vulchanova *et al.*, 1996, 1997, 1998; Bradbury et al., 1998; Le et al., 1998b), and P2X₂- and P2X₃ir are eliminated by dorsal root rhizotomy (Vulchanova et al., 1997, 1998; Bradbury et al., 1998), suggesting transport from DRG neurons. P2X₃-ir is localized in the inner part of lamina II (Vulchanova et al., 1997, 1998; Bradbury et al., 1998; Llewellyn-Smith and Burnstock, 1998; Ramer et al., 2001), which corresponds to the projection territories of IB4-, Ret-, and, in part, TRPV1-positive sensory neurons (Molliver et al., 1997; Vulchanova et al., 1998; Guo et al., 1999), consistent with the colocalization in the cell bodies of sensory neurons. P2X₃ in the dorsal horn could be delivered predominantly from IB4-positive sensory neurons (Vulchanova et al., 2001; Nakatsuka et al., 2003; Tarpley et al., 2004). An electron microscopic study, together with the above evidence, has revealed the presynaptic localization of the P2X₃ receptor (Llewellyn-Smith and Burnstock, 1998). A subpopulation of sensory neurons have $P2X_{2/3}$ receptors, but the colocalization of $P2X_2$ -ir and $P2X_3$ -ir seen in DRG neurons is rarely observed in the dorsal horn (Vulchanova et al., 1997). This could be simply due to technical limitations of immunostaining methods but is not likely the case because an overlap between $P2X_2$ - and $P2X_3$ -ir is seen in the nucleus of the solitary tract where the central terminals of NG neurons are located (Vulchanova et al., 1997). The reason why $P2X_2$ and $P2X_3$ are rarely colocalized in the dorsal horn remains unknown, but it is possible that they have different mechanisms for transporting protein to either peripheral or central sites at the axon branch point in the DRG, as has been suggested in other proteins (Garcia-Anoveros et al., 2001; Ji et al., 2002).

P2X receptors located at central terminals of sensory neurons in the dorsal horn could be functional and play roles in regulating neurotransmitter release presynaptically. Studies using spinal cord slices and cocultures of DRG neurons and dorsal horn neurons have shown that activating P2X receptors by ATP or $\alpha\beta$ meATP increases glutamatergic spontaneous and/or miniature excitatory postsynaptic currents (sEPSCs or mEPSCs, respectively) in dorsal horn neurons (Gu and MacDermott, 1997; Li et al., 1998; Labrakakis et al., 2000; Nakatsuka and Gu, 2001; Nakatsuka et al., 2002, 2003), both of which are sensitive to PPADS (Gu and MacDermott, 1997; Li et al., 1998; Nakatsuka and Gu, 2001; Nakatsuka et al., 2003). The increase in both mEPSCs and sEPSCs is mainly transient in lamina II neurons (Nakatsuka et al., 2003) and long lasting in lamina V neurons (Nakatsuka and Gu, 2001; Nakatsuka et al., 2002, 2003; Tsuzuki et al., 2003). This heterogeneity suggests that the P2X receptor subtypes involved might be different. While the transient increase in lamina II neurons is inhibited by PPADS and TNP-ATP and is eliminated by the removal of P2X₃-expressing

terminals in the superficial dorsal horn, the long-lasting modulation is not altered by such treatments (Nakatsuka *et al.*, 2003; Tsuzuki *et al.*, 2003). These lines of evidence suggest that P2X₃ receptors would be involved in the transient modulation of glutamate release in lamina II neurons and P2X receptors other than P2X₃ could be the likely candidates for the persistent increase in lamina V neurons (Nakatsuka *et al.*, 2003; Tsuzuki *et al.*, 2003), but these issues remain unsolved. These P2X receptors may be activated by endogenous ATP released from presynaptic terminals in an autocrine and/or paracrine fashion. In lamina II, PPADS can alter the ratio of paired-pulseevoked EPSCs (Nakatsuka and Gu, 2001). In lamina V neurons, $A\delta$ fiberstimulated EPSCs are potentiated when the breakdown of endogenous ATP is prevented by ARL 67156, an inhibitor of ecto-ATPase. Furthermore, PPADS increases the failure rate of the evoked EPSCs. Thus, ATP signaling via P2X receptors at central terminals may play a role in the glutamatergicdependent synaptic plasticity in the dorsal horn (Gu, 2003).

P2X receptors in the dorsal horn also control an inhibitory neurotransmission. ATP facilitates GABAergic/glycinergic inhibitory postsynaptic currents (IPCSs) in rat dorsal horn neurons in culture (Jo and Schlichter, 1999; Hugel and Schlichter, 2000). In mechanically dissociated rat lamina II neurons with native presynaptic nerve endings attached, the frequency of glycinergic miniature IPSCs is increased by ATP, and this increase is prevented by suramin and PPADS (Rhee et al., 2000; Jang et al., 2001). In lamina V neurons in dorsal horn slices, activating P2X receptors by $\alpha\beta$ meATP increases both the frequency and amplitude of spontaneous IPSCs but not those of miniature IPSCs (Nakatsuka et al., 2002), suggesting that presynaptic P2X receptors at primary afferent central terminals activate inhibitory interneurons, presumably via increasing glutamate release and thereby recruit inhibitory inputs on lamina V neurons. Thus, the ability of ATP to regulate sensory inputs and dorsal horn neuron excitability might be dependent on the sites of ATP action and the balance between its effects on excitatory and inhibitory terminals (Gu, 2003).

The accumulation of $P2X_3$ -ir proximal to the ligation site of sciatic nerve (Vulchanova *et al.*, 1998) or inferior alveolar nerve (Eriksson *et al.*, 1998), combined with evidence of $P2X_3$ expression in sensory neurons whose axons project to the periphery, such as skin (Cook *et al.*, 1997; Bradbury *et al.*, 1998), suggested that $P2X_3$ is transported to the periphery as well. $P2X_3$ -ir is found in the peripheral nerve endings of sensory neurons in the glabrous skin (Vulchanova *et al.*, 1998), cornea (Vulchanova *et al.*, 1998), tongue (Bo *et al.*, 1999), urinary bladder (Cockayne *et al.*, 2000; Yiangou *et al.*, 2001), and tooth pulp (Cook *et al.*, 1997; Renton *et al.*, 2003; Ichikawa and Sugimoto, 2004). Both *in vitro* and *in vivo* studies have shown that the excitation of primary sensory fibers by topical application of ATP or

αβmeATP in rats (Dowd *et al.*, 1998; Hamilton *et al.*, 2001) is inhibited by suramin or PPADS (Dowd *et al.*, 1998). The responding fibers are predominantly nociceptors, in particular C-mechanoheat polymodal nociceptors (Hamilton *et al.*, 2001). Excitation of human nociceptors by ATP was also observed *in vivo* (Hilliges *et al.*, 2002). Thus, P2X receptors at peripheral endings might be functional and activating these causes the excitation of a subpopulation of sensory neurons. However, the failure of P2X antagonists to modulate the responses to noxious thermal and mechanical stimuli under normal conditions (Tsuda *et al.*, 2000; Jarvis *et al.*, 2002; Dai *et al.*, 2004) also indicated that P2X receptors on peripheral endings could not mediate the excitation of sensory fibers evoked by those stimuli, although P2X₃ could be necessary for non-noxious "warm" stimuli (Souslova *et al.*, 2000). One prediction, therefore, is that ATP signaling via P2X receptors may be activated in certain specific situations and contribute to the pain sensation under such situation as described in the following section.

VII. ACTIVATING P2X RECEPTORS CAUSES AND MODULATES PAIN SENSATION

All cells contain millimolar levels of ATP in their cytosol and can release ATP into the extracellular milieu when tissues are damaged (Burnstock and Wood, 1996). A potential hypothesis is that ATP is a chemical trigger for pain following peripheral tissue damage. In a coculture of sensory neurons with keratinocytes, P2X₃- and P2X_{2/3}-like inward currents were evoked in sensory neurons when nearby keratinocytes were damaged (Cook and McCleskey, 2002). In vivo behavioral studies using animal models of tissue injury-induced pain have shown that pain responses (hindpaw lifting and licking) by injection of formalin into the hindpaw are reduced either by blocking P2X receptors pharmacologically at the damaged area (Sawynok and Reid, 1997; Jarvis et al., 2001; McGaraughty et al., 2003), or by reducing the expression of P2X₃ receptor molecularly (Honore et al., 2002), or genetically (Cockayne et al., 2000; Souslova et al., 2000). Furthermore, activating P2X₃ receptors by injecting their agonists into the hindpaw causes similar nocifensive responses (Bland-Ward and Humphrey, 1997; Hamilton et al., 1999; Cockayne et al., 2000; Tsuda et al., 2000; Jarvis et al., 2001; Honore et al., 2002; McGaraughty et al., 2003), which are also inhibited by P2X antagonists (Jarvis et al., 2001; McGaraughty et al., 2003), P2X₃ antisense (Honore et al., 2002; Tsuda et al., 2002), and by $P2X_3$ gene deletion (Cockayne et al., 2000). This pain behavior is also eliminated in neonatal capsaicin-treated rats (Hamilton et al., 1999; Tsuda et al., 2000) (Fig. 1C) that have dramatically reduced $P2X_3$ -ir (Vulchanova *et al.*, 1998) and P2X₃-mediated currents in their DRG neurons (Tsuda et al., 2000) (Fig. 1A), suggesting that P2X₃ receptors on peripheral nerve endings would serve as a pain generator by receiving the ATP released from damaged tissue. A study has also suggested that downregulation of the P2X₃mediated pain signaling system may be responsible for the predisposition for low sensitivity to tissue injury pain observed in the A/J inbred mouse strain (Tsuda et al., 2002). In addition to nocifensive behavior, P2X agonist can produce hypersensitivity to noxious heat (i.e., thermal hyperalgesia) (Hamilton et al., 1999; Tsuda et al., 2000) and mechanical stimuli (Barclay et al., 2002). Those are also suppressed by P2X₃ antisense (Barclay et al., 2002) (our unpublished data) and in capsaicin-treated rats (Tsuda et al., 2000), as is nocifensive behavior, suggesting the involvement of $P2X_3$. There is another phenotype of pain behavior elicited by activating P2X receptors, namely mechanical allodynia (Tsuda et al., 2000; Dorn et al., 2004). The P2X receptors involved in allodynia are, different from those involved in other types of pain. This is because the allodynia remains in neonatal capsaicin-treated rats (Tsuda et al., 2000) (Fig. 1D) whose DRG neurons show $P2X_{2/3}$ -like currents as do control neurons (Fig. 1B) and, by contrast, show the reduced P2X₃-like currents (Tsuda et al., 2000) (Fig. 1A). Furthermore, the $\alpha\beta$ meATP-induced allodynia is suppressed by an



FIGURE 2 The effect of antisense oligodeoxynucleotide targeting P2X₃ or P2X₂ receptor on the $\alpha\beta$ meATP-induced mechanical allodynia. The mechanical allodynia caused by intraplantar injection of $\alpha\beta$ meATP was significantly inhibited by repeated intrathecal pretreatment with P2X₃ antisense (A: **p < 0.01 versus PBS-treated group) or with P2X₂ antisense (B: **p < 0.01 versus PBS-treated group). Neither P2X₃ nor P2X₂ missense affect the $\alpha\beta$ meATP-induced mechanical allodynia. Reprinted from Inoue *et al.* (2003).

antisense targeting the P2X₃ (Fig. 2A) or P2X₂ receptor (Fig. 2B). By contrast, nocifensive behavior and thermal hyperalgesia are suppressed by P2X₃ antisense (Inoue *et al.*, 2003) or siRNA (Dorn *et al.*, 2004) but not by P2X₂ antisense (Inoue *et al.*, 2003). These suggest the possibility that activation of P2X_{2/3} receptors in capsaicin-insensitive fibers produces mechanical allodynia.

In the dorsal horn in vivo, P2X receptors could enhance the excitatory inputs of noxious heat and mechanical or innocuous mechanical stimuli. Activating P2X receptors by means of intrathecal administration of $\alpha\beta$ meATP produces a transient thermal and mechanical hyperalgesia (Tsuda et al., 1999a; Okada et al., 2002) and mechanical allodynia (Fukuhara et al., 2000), both of which are inhibited by PPADS or TNP-ATP (Tsuda et al., 1999a; Fukuhara et al., 2000). These hypersensitivities appear to be associated with enhanced presynaptic glutamate release, which in turn activates NMDA receptors in the dorsal horn neurons (Tsuda et al., 1999a; Fukuhara et al., 2000). A role for endogenous ATP and P2X receptors in the spinal cord has also been suggested. In models of chemical-induced nociception, P2X antagonists injected intrathecally produce pronounced antinociceptive effects on the pain responses caused by formalin (Tsuda et al., 1999b; McGaraughty et al., 2003), capsaicin (Tsuda et al., 1999b), prostaglandin E₂ (Fukuhara *et al.*, 2000), platelet-activating factor (Morita et al., 2004), and bee venom (Zheng and Chen, 2000).

P2X receptors in the brain have been shown to be involved in pain processing, but their activation could result in an analgesic effect. Intracerebroventricular administration of ATP, $\alpha\beta$ meATP or 3'-O-(4-benzoyl)benzoyl ATP (BzATP) produces an antinociceptive effect on noxious mechanical stimuli (Fukui *et al.*, 2001), while neither $\beta\gamma$ meATP nor UTP affects mechanical sensitivity. It thus appears that brain P2X₃ receptors are involved in the antinociception, but further studies are needed to identify the receptor subtypes.

VIII. PERSISTENT PERIPHERAL INFLAMMATION ENHANCES P2X RECEPTOR EXPRESSION AND THEIR FUNCTION, THEREBY CAUSING PAIN

P2X-mediated nocifensive behavior is greatly enhanced in rats whose hindpaw is inflamed by carrageenan or by ultraviolet irradiation (Hamilton *et al.*, 1999). A similar enhanced sensitivity to ATP is also observed in humans (Bleehen and Keele, 1977; Coutts *et al.*, 1981; Hamilton *et al.*, 2000). In an *in vitro* skin–nerve preparation, activating P2X receptors produced the excitation of C-mechanoheat polymodal nociceptors, which are enhanced in preparations of inflamed skin (Hamilton et al., 2001). Together with the evidence that the levels of ATP in inflamed tissues are elevated (Ryan et al., 1991; Park et al., 1996), it is thus predicted that P2X receptors on peripheral nerve endings in inflamed areas would be activated, thereby modulating pain processing. P2X antagonists reduce the heightened sensitivity to noxious (hyperalgesia) or innocuous stimuli (allodynia) caused by complete Freund's adjuvant (CFA) (Dell'Antonio et al., 2002a,b; Jarvis et al., 2002; McGaraughty et al., 2003; Dai et al., 2004), an animal model of inflammatory pain. P2X₃ antisense also reversed and prevented hyperalgesia in a CFA model (Barclay et al., 2002; Honore et al., 2002). Similar results were observed in studies using the selective P2X₃ and P2X_{2/3} antagonist A-317491 (Jarvis et al., 2002; McGaraughty et al., 2003; Wu et al., 2004). Mutant mice lacking $P2X_3$ receptors, however, showed, even enhanced, thermal hyperalgesia in a CFA model (Souslova et al., 2000). The discrepancy remains unresolved, but it might be due to unknown compensatory effects resulting from P2X₃ gene disruption. It is of particular interest to note that neither A-317491 nor P2X₃ antisense reduces the heightened pain sensitivity by carrageenan, another inflammatory pain model (Honore et al., 2002; Jarvis et al., 2002). P2X₃knockout mice also display the carrageenan-induced hyperalgesia, as do wild-type mice (Souslova et al., 2000). Since the inflammatory pain in the CFA model persists much longer than that in the carrageenan model, P2X receptors may thus play more important roles in chronic than acute inflammatory pain. This view is supported by evidence that persistent inflammation by CFA is accompanied by an upregulation of both $P2X_2$ and $P2X_3$ receptors in sensory neurons (Xu and Huang, 2002). The upregulation might lead to an increase in both transient and persistent ATP currents in DRG neurons ipsilateral to the inflamed hindpaw by CFA. However, it seems unlikely that the phenotype of P2X receptors in the DRG neurons of the inflamed side is changed because the pharmacological profiles of both currents are similar to those in control DRG neurons. The increase in ATP responses in inflamed DRG neurons generates large depolarizations (Xu and Huang, 2002). Heightened responses via P2X receptors in DRG neurons in vitro are also observed in P2X receptors at the peripheral terminals in vivo. Dai et al. (2004) have shown that activating P2X receptors on peripheral endings produces an activation of extracellular signal-regulated protein kinase (ERK), a member of mitogen-activated protein kinases (MAPKs), in DRG neurons only under the situation in which peripheral tissues are inflamed. The majority of activated ERK-positive DRG neurons express $P2X_3$ receptors. The level of activated ERK is markedly enhanced by mechanical stimulation given to the inflamed hindpaw, and the enhancement (especially in large-sized neurons) requires the activation of P2X receptors, presumably by endogenous ATP at the periphery. ATP is actually released from keratinocytes by mechanical stimulation (Koizumi et al., 2004). Moreover, TNP-ATP, but not Ip_5I , reduces CFA-induced mechanical allodynia (Dai et al., 2004) suggesting the predominant role of $P2X_{2/3}$ receptors. These lines of evidence suggest that persistent peripheral inflammation causes both quantitative and qualitative upregulation of P2X receptors in sensory neurons, which in turn leads to heightened responses of P2X receptors by released endogenous ATP, and thereby contributes to inflammatory pain hypersensitivity. The cellular mechanisms by which the expression and function of P2X receptors are upregulated in sensory neurons are still unknown but several possibilities are considered (Fig. 3). Since peripheral inflammation increases the levels of various inflammatory mediators in the inflamed area (Julius



FIGURE 3 Schematic illustration of possible mechanisms by which P2X receptors on peripheral nerve endings modulate pain signaling evoked by mechanical stimulation following persistent peripheral inflammation. The expression of P2X receptors (i.e., $P2X_2$ and $P2X_3$) is increased in sensory neurons following peripheral inflammation, activation of which results in generation of large depolarizations in sensory neurons. Peripheral inflammation leads to the release of inflammatory mediators such as bradykinin and prostaglandins. These mediators activate their receptors and may then enhance function of P2X receptors via intracellular signaling events such as protein kinase C (PKC) and A (PKA). Activating sensitized P2X receptors by ATP, which is released from skin cells by mechanical stimulation causes the excitability of sensory neurons, which in turn leads to increased sensory pain signaling to the dorsal horn.

and Basbaum, 2001; Scholz and Woolf, 2002), there may be an interaction between P2X receptors and these mediators that produces the functional upregulation of P2X receptors. P2X-mediated responses are enhanced by substance P (Hu and Li, 1996; Paukert et al., 2001), neurokinin B (Wang et al., 2001), prostaglandin E₂ (Hamilton et al., 1999), protons (Li et al., 1997), and bradykinin (Paukert et al., 2001). Some of the regulations are mediated through the phosphorylation of P2X receptors by protein kinases (Paukert et al., 2001; Wang et al., 2001). For the upregulation of P2X receptor expression, growth factors could be candidates. Ramer et al. (2001) have shown that GDNF treatment increases $P2X_3$ expression. NGF also increases the expression of $P2X_3$ in DRG neurons and induces new expression in some DRG neurons. The fact that the NGF levels are dramatically elevated following peripheral inflammation suggests that de novo expression of $P2X_3$ by NGF in TrkA-positive DRG neurons could contribute to the enhanced ATP signaling via P2X receptors. Chessell et al. (2005) have shown that increases in proinflammatory mediators, in particular interleukin-1 β , in the inflamed hindpaw are reduced in mutant mice lacking $P2X_7$ receptors. Furthermore, the $P2X_7$ knockout mice show a reduced pain hypersensitivity caused by CFA. It seems that $P2X_7$ receptors which are known to be expressed in immune cells (Ralevic and Burnstock, 1998) but not in sensory neurons (Chessell et al., 2005; Kobayashi et al., 2005) may be one of triggers to increase inflammatory mediators in the inflamed tissues and may also contribute to inflammatory pain.

IX. P2X RECEPTORS IN SENSORY NEURONS HAVE CRUCIAL ROLES IN NEUROPATHIC PAIN

Peripheral nerve injury leads to intractable neuropathic pain that is often resistant to most current treatments (Woolf and Mannion, 1999; Woolf and Salter, 2000; Scholz and Woolf, 2002). Accumulating evidence has suggested that molecular and cellular alterations in primary sensory neurons as well as in the dorsal horn after nerve injury have an important role in the pathogenesis of neuropathic pain. Those alterations include P2X receptors in sensory neurons. Transection of the sciatic nerve (axotomy model of neuropathic pain) produces a marked reduction of P2X₃-ir in injured DRG (Bradbury et al., 1998). In contrast, an increase in the number of P2X₃-ir-positive DRG neurons is seen following partial nerve injury by chronic constriction of the sciatic nerve (Novakovic et al., 1999) or of the inferior alveolar nerve (Eriksson et al., 1998), both of which are also models of neuropathic pain. The difference in the change of $P2X_3$ expression might be due to the type of nerve injury (that is, complete or partial), since a study examining the expression of activating transcription factor 3 (ATF3), a neuronal injury marker, has shown that there is an increase in the expression of $P2X_3$ in

DRG neurons whose axons had been spared following partial nerve injury (Tsuzuki *et al.*, 2001). However, in an L5 spinal nerve injury model, the expression of P2X₃ was, as expected, markedly decreased in L5 DRG neurons whose axons had been completely injured but, however, not changed in L4 DRG neurons whose axons had been spared (Fukuoka *et al.*, 2002). Thus, the increase in the P2X₃ expression in spared DRG neurons could require neighboring injured neurons within the DRG. Besides P2X₃, the regulation of other P2X receptors also changes following nerve injury. mRNAs of P2X₅ and P2X₆ are increased and decreased, respectively, in the injured DRG (Kim *et al.*, 2003). P2X₂ mRNA is unchanged, but there is a marked increase in the number of P2X₂-ir–positive neurons in the injured DRG (Kim *et al.*, 2003). These findings suggest posttranscriptional alterations as well as transcriptional changes, and raise the possibility that the composition of P2X subunits in P2X receptor may be changed in affected sensory neurons following nerve injury.

Among the seven P2X receptors, P2X₃ would be a key subunit in neuropathic pain. Reduction in P2X₃ expression in DRG neurons by P2X₃ antisense or siRNA prevents the development of mechanical hypersensitivity by partial nerve injury of the sciatic nerve (Barclay et al., 2002; Honore et al., 2002; Dorn et al., 2004). P2X₃ antisense also reverses neuropathic pain hypersensitivity (Barclay et al., 2002; Honore et al., 2002), which reemerges within several days after the cessation of treatment with $P2X_3$ antisense (Honore *et al.*, 2002), suggesting that the $P2X_3$ receptor also has an ongoing role. This is substantially supported by a finding that A-317491 reverses allodynia and hyperalgesia after nerve injury (Jarvis et al., 2002; McGaraughty et al., 2003). It is of particular interest to note that reversing or preventing neuropathic pain behaviors by P2X₃ antagonists or antisense is observed not only in partial nerve injury models that are accompanied by the upregulation of P2X₃ expression in DRG neurons (Novakovic et al., 1999) but also in spinal nerve injury models that decrease the expression of P2X₃ receptors in L5 DRG neurons without affecting their expression in L4 DRG neurons (Fukuoka et al., 2002; Kage et al., 2002; Kim et al., 2003). These findings thus indicate that $P2X_3$ could contribute to the pathogenesis of neuropathic pain even if P2X₃ expression in sensory neurons is not upregulated.

There are several possible mechanisms by which P2X receptors (especially P2X₃) contribute to neuropathic pain (Fig. 4). Based on evidence showing the presence of P2X₃ receptors on peripheral nerve endings (see the preceding section) and because ATP is released from peripheral tissues including skin (Burnstock and Wood, 1996; Cook and McCleskey, 2002; Koizumi *et al.*, 2004), P2X₃ receptors in the periphery could be involved. This might, however, not be the case, since blocking P2X₃ receptors on peripheral nerve endings by locally injection with A-317491 does not change the allodynia



FIGURE 4 Schematic illustration of possible mechanisms by which P2X receptors on cell body in the sensory ganglia and on central terminal in the dorsal horn modulate pain signaling following peripheral nerve injury. The expression of P2X receptors is changed in sensory neurons following peripheral nerve injury. Cell body of sensory neurons may express functional P2X receptors on its cell surface presumably via Ca²⁺/calmodulin protein kinase II (CaMKII). ATP released from sympathetic nerve terminals that sprout into sensory ganglia may then activate the P2X receptors. The P2X-mediated excitability of sensory neurons from sympathetic nerve leads to release of glutamate and ATP in the dorsal horn, and activating presynaptic P2X receptors also enhances glutamate release. Signals from activated P2X receptors on central terminals of sensory neurons may cause changes in excitatory or inhibitory synaptic transmission of dorsal horn neurons. The enhanced inhibitory transmission may cause excitability of dorsal horn neurons under neuropathic pain state because of the abnormal anion gradient in the dorsal horn neurons resulting from downregulating the potassium-chloride exporter KCC2.

occurring after partial nerve injury or spinal nerve injury (McGaraughty et al., 2003). However, a putative role of the $P2X_3$ receptor accumulation in the proximity of the injury site of sensory fibers (Eriksson et al., 1998; Vulchanova et al., 1998) cannot be ruled out. In the dorsal horn, P2X receptors at central terminals could be involved since an intrathecal injection of A-317491 reverses allodynia (McGaraughty et al., 2003). Endogenous ATP may be released from central terminals of sensory fibers by excess stimulation derived by nerve injury (Nakatsuka and Gu, 2001), by dorsal horn interneurons (Jo and Schlichter, 1999), or by activated dorsal horn astrocytes (Fam et al., 2000). The released ATP then activates P2X receptors at central terminals of the primary afferents and thereby enhances the release of glutamate, which results in an increase in the excitatory transmission in dorsal horn neurons (Fig. 4). An additional possibility is that, based on the evidence that peripheral nerve injury changes the normally GABAergic/ glycinergic-mediated inhibitory transmission to excitatory in lamina I neurons by an abnormal anion gradient resulting from downregulation of the potassium-chloride exporter KCC2 (Coull et al., 2003), an enhanced inhibitory GABAergic/glycinergic transmission by activating P2X receptors in the normal state (Jo and Schlichter, 1999; Hugel and Schlichter, 2000; Rhee et al., 2000; Jang et al., 2001; Nakatsuka et al., 2002) may lead to an excitability of dorsal horn neurons in a neuropathic pain state, which may be responsible for the reversal of neuropathic pain by P2X antagonists (Fig. 4). However, there are also controversial results showing that PPADS or suramin injected intrathecally fails to reverse neuropathic pain (Liu and Tracey, 2000), nor do they suppress C- and A β -fiber-evoked neuronal responses in the deep dorsal horn in a model of neuropathic rats (Stanfa et al., 2000). The reason for the discrepancy remains unknown, but a complicating element could be their selectivity to P2X receptors.

An alternative mechanism could be that new pain signaling pathways utilizing ATP and P2X receptors are developed in sensory neurons (Fig. 4). Generally, sensory neurons do not receive synaptic inputs to their cell body, but following peripheral nerve injury sympathetic axons sprout into the DRG and form abnormal terminal arborizations around some DRG neurons, which is thought to underlie the sympathetically maintained component of neuropathic pain in humans (Ramer *et al.*, 1999). Considering the fact that ATP is released from sympathetic nerve endings (Burnstock and Wood, 1996;

Also, shown is $P2X_4$ receptor on activated microglia neighboring dorsal horn neurons. Potential mechanisms of enhanced excitability are illustrated on the left side and those of enhanced inhibitory transmission, which in turn causes excitability in the dorsal horn neurons are shown on the right side.



FIGURE 5 Contribution of $P2X_4$ receptors in dorsal horn microglia to expression of neuropathic pain. Intrathecal administration of TNP-ATP but not PPADS reverses tactile

Dunn et al., 2001), ATP may activate P2X receptors expressed on the cell body of DRG neurons. In this pathway, the expression of functional P2X receptors on the surfaces of sensory neuron cell bodies is needed. However, in vitro preparations of intact (not dissociated) DRG with both attached dorsal root and spinal nerve from normal rats, only few neurons or fibers respond to ATP or $\alpha\beta$ meATP (Stebbing *et al.*, 1998; Zhou *et al.*, 2001), suggesting that sensory neuronal cell bodies could not express P2X receptors on their cell surface, or even if expressed, the P2X receptors could be dysfunctional. However, after peripheral nerve injury, the DRG whose axons had been injured do respond to ATP or $\alpha\beta$ meATP (Zhou *et al.*, 2001). Furthermore, a study has shown that electrical stimulation of DRG neurons increases the expression of P2X₃ receptors on the cell surface via the activation of $Ca^{2+}/$ calmodulin dependent protein kinase II (Xu et al., 2004). Therefore, after peripheral nerve injury, P2X receptors on the cell surface may be increased and/or become functional. Therefore, it will be of particular interest to investigate the roles of P2X receptors in the sympathetic nerve-dependent excitation of sensory neurons using selective P2X antagonists or knockout animals.

X. P2X₄ RECEPTORS IN SPINAL MICROGLIA ARE ESSENTIAL FOR NEUROPATHIC PAIN

We revealed that the P2X₄ receptor in the spinal cord is required for the expression of neuropathic pain (Tsuda *et al.*, 2003). Importantly, the expression of P2X₄ in the spinal cord is highly restricted to activated microglia after nerve injury. Microglia represent 5–10% of glial population in the CNS and are often considered resident macrophages. In adults, microglia are distributed through-

allodynia caused by L5 spinal nerve injury (A). The line graphs show the effects of intrathecal administration of TNP-ATP (30 nmol; black squares) and PPADS (30 nmol; black triangles) on the decrease in paw withdrawal threshold (g) 7 days after nerve injury (^{##}p < 0.01 and ^{###}p < 0.001 versus PBS-treated group; black circles). Panel B shows the anti-allodynic effect (mean \pm SEM) of TNP-ATP 7 days after nerve injury (^{##}p < 0.01 and ^{####}p < 0.001 versus PBS-treated group; black circles). Panel B shows the anti-allodynic effect (mean \pm SEM) of TNP-ATP 7 days after nerve injury (^{##}p < 0.01 and ^{####}p < 0.001 versus PBS-treated group). Anti-allodynic effect (%) = 100 × (test value – pre-injection value)/(15.1 g – pre-injection value). An antisense oligodeoxynucleotide (AS) targeting P2X₄ receptor prevents the nerve injury-induced allodynia (C: ##p < 0.01 versus missense [MS]-treated group). The protein level of P2X₄ in the spinal cord from AS-treated rats was clearly lower than that from MS-treated rats (see insert). Western blot analysis of P2X₄ protein detected by P2X₄ antibody in the membrane fraction from the spinal cord ipsilateral to the nerve injury at different day (D). Double immunofluorescent labels of P2X₄ with NeuN (E), left, a marker of neurons, GFAP (E), middle, a marker of astrocytes, and OX42 (E), right, a marker of microglia. Most P2X₄-positive cells (arrowheads) are double-labeled with OX42 (E), right (arrowheads). Scale bars: 50 µm. Reprinted from Tsuda *et al.* (2003).

out CNS and, unlike macrophages, have a small soma bearing thin and branched processes under normal conditions. Such microglia are said to be "resting," but resting microglia are not dormant, rather, they act as sensors for a range of stimuli that threaten physiological homeostasis, including CNS trauma, ischemia, infection, and peripheral nerve injury. After peripheral nerve injury, activated microglia in the dorsal horn dramatically express P2X₄ receptors (Tsuda *et al.*, 2003) but dorsal horn neurons or astrocytes do not (Figs. 5D and E). Acute pharmacological blockade of P2X₄ receptors reverses the established neuropathic pain, and an antisense targeting P2X₄ receptors in microglia is necessary for sustaining neuropathic pain. Moreover, spinal administration of P2X₄-stimulated microglia causes allodynia and therefore P2X₄ receptor activation in microglia is not only necessary but also is sufficient to cause neuropathic pain.

ATP plays not only as a neurotransmitter in the dorsal horn but also as an activator of glial cells including microglia. ATP stimulates the release of plasminogen (Inoue *et al.*, 1998), tumor necrosis factor- α (TNF- α) (Hide *et al.*, 2000; Suzuki *et al.*, 2004), and interleukin-6 (IL-6) (Shigemoto-Mogami *et al.*, 2001) from microglia. Furthermore, several cytokines have been reported to alter synaptic transmission in the CNS including the spinal cord (Kerr *et al.*, 1999; Thompson *et al.*, 1999; Vikman *et al.*, 2003). Moriguchi *et al.* (2003) have shown in acute cortical slices that the application of a microglial-conditioned medium potentiates NMDA receptor-mediated post-synaptic responses. It thus appears that ATP released from central terminals of primary afferents (and/or dorsal horn astrocytes) also activates P2X₄ receptors on dorsal horn microglia, thereby releasing diffusible microglial factors, such as cytokines, which in turn lead to changes in the properties of the spinal pain processing network to bring about peripheral nerve injury-induced pain hypersensitivity (Tsuda *et al.*, 2005) (Fig. 4).

XI. CONCLUDING REMARKS

As we have described here, ATP might be an important player not only for tissue injury-induced pain but also pathological chronic pain via P2X receptors expressed on peripheral and/or central terminals, or on the cell body of sensory neurons. Efforts focusing on the distribution and electrophysiological properties of P2X receptors in sensory neurons have revealed that P2X₃ and P2X_{2/3} receptors might be the predominant functional P2X receptors. Activating P2X receptors at sensory nerve endings at both the periphery and the dorsal horn produces action potentials and an increase in sensory synaptic strength, respectively, both of which have important im-

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plications in pain generation and modulation. *In vivo* behavioral studies have strongly supported this view and revealed an ongoing role in addition to the developmental role of P2X receptors in pathological pain. Furthermore, P2X₄ receptors on dorsal horn microglia also participate in the pathogenesis of neuropathic pain. Evidence has also strongly suggested an important role of P2X receptors in visceral pain (Cockayne *et al.*, 2000; Burnstock, 2001; Chizh and Illes, 2001; Burnstock, 2002; Jarvis *et al.*, 2002; Cervero and Laird, 2004). Together with the evidence that neither antagonists, antisense nor siRNA targeting P2X receptors affect the basal responses to physiological pain, selective antagonists for P2X receptors could be potent therapeutic drugs for pathological pain without affecting physiological pain.

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CHAPTER 10

Voltage-Gated Sodium Channels and Neuropathic Pain

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- I. Overview
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I. OVERVIEW

Voltage-gated sodium channels (VGSCs) play an important role in the generation of neuronal activity in not only normal but also pathological conditions. Many of the identified subtypes of VGSCs are found in sensory neurons in the dorsal root ganglion (DRG). Based on their sensitivity to tetrodotoxin (TTX), they are divided into TTX-sensitive (TTXs) or TTX-resistant (TTXr) types. In neuropathic pain, ectopic discharges originating from injured sensory neurons are considered the culprit in maintaining central sensitization, thus, they cause hyperalgesia and allodynia. Ectopic discharges are blocked by TTX at doses that are comparable to TTXs subtypes. The same low doses of TTX applied to the DRG of the injured segment significantly reduced pain behaviors in neuropathic rats. Data thus suggest that TTXs subtypes of VGSCs play an important role in the generation

of both ectopic discharges and neuropathic pain. Analysis of mRNA of the TTXs subtypes of VGSCs in the DRG show that $Na_v1.3$ is upregulated after spinal nerve ligation, suggesting its potential importance in the generation of ectopic discharge and neuropathic pain. On the contrary, TTXr VGSC $Na_v1.8$ is expressed preferentially in small-diameter DRG neurons, presumably nociceptors. After nerve injury, the expression of $Na_v1.8$ and TTXr current are upregulated in neighboring uninjured but sensitized sensory neurons. The data thus suggest that $Na_v1.8$ is likely involved in the generation of neuropathic pain through uninjured but sensitized sensory neurons.

II. INTRODUCTION

VGSCs play a fundamentally important role in the excitability of almost all neurons in the central and peripheral nervous systems. They are located in the plasma membrane and mediate the influx of sodium ions into the cell in response to membrane depolarization. Sodium influx results in the generation of the action potential. Ten distinct pore-forming α subunits of VGSCs have been identified in vertebrates, and many of these are localized in DRG neurons (Goldin *et al.*, 2000).

Peripheral nerve injury can result in neuropathic pain in which normally painful peripheral stimuli produce severe pain (hyperalgesia), and normally nonpainful stimuli produce pain (allodynia). This condition involves an abnormal central processing state, central sensitization, which is initiated and maintained by abnormal peripheral input (Gracely *et al.*, 1992). Peripheral nerve injuries are likely to include mechanical damage and local inflammation, both of which lead to the development of pathological afferent activity. This pathological afferent activity includes ectopic discharges from axotomized afferents and spontaneous activity of sensitized intact nociceptors. Blocking the entrance of ectopic discharges into the spinal cord by dorsal rhizotomy reduces neuropathic pain behaviors, suggesting that at least part of the pain behaviors is maintained by ectopic discharges (Chung and Chung, 2002).

It has been proposed that dynamic changes in VGSCs after nerve injury play an important role in generating ectopic discharges and spontaneous activity of sensory neurons. This chapter discusses evidence supporting the hypothesis that alterations of one or two subtypes of VGSCs in axotomized afferents contribute to the generation and maintenance of neuropathic pain. A detailed review of VGSCs of intact nociceptors that are critically involved in inflammatory and neuropathic pain may be found elsewhere (Lai *et al.*, 2004).

III. THE STRUCTURE OF VGSCs

VGSCs are the key element of excitable cells in the generation and transmission of electrical activity. Genes encoding ten distinct α subunits of VGSCs have been identified in vertebrates and have been classified into subfamilies under a unified nomenclature, from Nav1.1 through Nav1.9 and Na_x (Goldin *et al.*, 2000). Each VGSC comprises a large pore-forming α subunit and one or more β subunits, however, a single α subunit constitutes a functional VGSC. The α subunit consists of four domains (D1–D4) connected by intracellular loops and each domain contains six transmembrane segments (S1-S6). The extracellular S5-S6 link for each domain contributes pore-lining residues with a selectivity filter (Catterall, 2000). Amino acids, such as serine (as in Nav1.8 and Nav1.9) and cysteine (as in $Na_v 1.5$), in the pore-lining segment of D1 cause the channel to be resistant to TTX (Satin et al., 1992; Akopian et al., 1996; Sivilotti et al., 1997; Cummins et al., 1999; Cantrell and Catterall, 2001). On the other hand, a residue with an aromatic side chain, such as phenylalanine or tyrosine, causes the channel to be sensitive to TTX (Satin *et al.*, 1992; Cantrell and Catterall, 2001). β subunits appear to serve a number of functions, including targeting and anchoring channels at specific sites in the plasma membrane and modulation of the gating properties of the α subunits (Isom, 2001). Genes encoding three β subunits have been identified (Goldin *et al.*, 2000).

IV. SUBTYPES OF VGSCs AND SODIUM CURRENTS IN SENSORY NEURONS

The cell-specific expression of VGSC subtypes is critical in determining the heterogeneity and functional specialization of primary sensory neurons that propagate sensory or nociceptive signals to the spinal cord and brain. Since many different types of primary sensory neurons exist in the DRG, the expression of subtypes of VGSCs among subpopulations of sensory neurons is heterogeneous (Black *et al.*, 1996). Some examples of these differential expressions include: the transcripts of Na_v1.1 are preferentially expressed in large neurons; Na_v1.6 and Na_x are high in most large neurons as well as many medium-sized neurons; and Na_v1.7, Na_v1.8, and Na_v1.9 are preferentially expressed in small neurons (Akopian *et al.*, 1996; Black *et al.*, 1996; Dib-Hajj *et al.*, 1998; Novakovic *et al.*, 1998; Cummins *et al.*, 1999; Gould III *et al.*, 2000; Djouhri *et al.*, 2003). Some sodium channels are developmentally regulated; thus, Na_v1.2, Na_v1.3, and Na_v1.5 are highly expressed in embryonic sensory neurons but are hardly detected in a small number of neurons in the adult DRG (Waxman *et al.*, 1994; Black *et al.*, 1996; Renganathan *et al.*, 2002; Chung *et al.*, 2003). In addition, sodium channel subtypes are differentially expressed at different sites of neurons. For example, $Na_v1.6$ is highly localized to nodes of Ranvier (Caldwell *et al.*, 2000); $Na_v1.7$ is preferentially expressed in axon terminals (Toledo-Aral *et al.*, 1997); $Na_v1.8$ is preferentially expressed in the cell body and possibly the terminal arbor; while $Na_v1.9$ is expressed throughout neurons that give rise to unmyelinated axons (Fjell *et al.*, 2000).

While there has been great progress in identifying and characterizing subtypes of sodium channels based on their molecular structures, physiological characterization of sensory neurons still largely depends on electrophysiological properties of action potentials and sodium currents of individual neurons. Based on their sensitivity to TTX, sodium currents are generally classified into two types: TTXs and TTXr (Ogata and Tatebayashi, 1993).

Only one type of TTXs current is recorded from primary sensory neurons and these currents can be blocked by TTX at concentration in the low-nanomolar range (<10 nM). The TTXs VGSCs tend to have a low threshold for activation, are rapidly activating and inactivating, but slow in recovering from inactivation (Ogata and Tatebayashi, 1993; Cummins and Waxman, 1997). Transcripts for four TTXs VGSCs, $Na_v1.1$, $Na_v1.6$, $Na_v1.7$, and Na_x , seem to account for most of the TTXs signal in adult rat DRG neurons (Waxman *et al.*, 1994; Black *et al.*, 1996; Dib-Hajj *et al.*, 1998; Kim *et al.*, 2001a).

TTXr currents are further divided into three types based on kinetics of activation and inactivation (Kostyuk et al., 1981; Caffrey et al., 1992; Elliott and Elliott, 1993; Akopian et al., 1996; Cummins et al., 1999): fast-inactivating $TTXr_{F}$, slow-inactivating $TTXr_{S}$, and persistent $TTXr_{P}$ currents. Of the three different types of TTXr currents, TTXrs is highly resistant to TTX $(>100 \mu M)$, has slow kinetics and a high-activation threshold, and is thought to contribute to the action potential inward current, and thus is responsible for the TTXr broad action potentials in nociceptive neurons (Akopian et al., 1996). The TTXr_P currents are unique, having high-TTX resistance (40–60 μ M), extremely low thresholds for activation (-90 \sim -70 mV) and an ultraslow inactivation rate; thus the persistent nature of the current may influence membrane potential in small DRG neurons (Cummins et al., 1999; Herzog et al., 2001). Sodium currents of TTXr_F have characteristics similar to those of TTXs currents, with TTX sensitivity in the $1-2 \mu M$ range. They can be recorded from most embryonic DRG neurons but are detectable in only 5% of small DRG neurons of adult rats (Satin et al., 1992; Renganathan et al., 2002). There has been compelling evidence to suggest that $Na_v 1.5$, $Na_v 1.8$, and $Na_v 1.9$ are responsible for $TTXr_F$, $TTXr_S$, and TTXr_P currents, respectively (Akopian et al., 1996; Cummins et al., 1999; Fjell et al., 2000; Renganathan et al., 2002).

V. TTXs VGSCs ARE IMPORTANT FOR ECTOPIC DISCHARGES AND NEUROPATHIC PAIN

The importance of VGSCs for the generation of ectopic discharges is supported by various studies. VGSCs accumulate at the neuroma of a cut sensory nerve where some ectopic discharges arise (Devor et al., 1989), and VGSC blockers silence ectopic discharges (Devor et al., 1992; Matzner and Devor, 1994). Furthermore, application of sodium channel blockers reduces neuropathic pain in humans (Chabal et al., 1992; Hunter and Loughhead, 1999) and diminishes pain behaviors in animal models of neuropathic pain (Abram and Yaksh, 1994; Chaplan et al., 1995; Abdi et al., 1998; Lyu et al., 2000). The critical role of TTXs subtypes for the generation of ectopic discharges became apparent, however, only after the TTX sensitivity of ectopic discharges was tested in an in vitro DRG recording system (Liu et al., 2001). In this in vitro DRG recording study, the ipsilateral L5 DRG along with the dorsal root and the ligated spinal nerve were removed from the L5 spinal nerve ligated (SNL) neuropathic rat and placed in a recording chamber, each part in a separate perfusion chamber. Single unit ectopic discharges were recorded from the teased dorsal root filaments. Sustained ectopic discharges were recorded from 13 h and afterward following spinal nerve ligation and most of these ectopic discharges originated from the DRG (Liu et al., 1999, 2001). When TTX was applied to the DRG, the site of origin of ectopic discharges, the ectopic discharges were significantly suppressed at the average TTX dose of 22 nM (Liu et al., 2001). This reduction is not due to conduction block, which required \sim 400 nM of TTX. The TTX dose required to reduce ectopic discharges is about two orders of magnitude lower than the TTX sensitivity of TTXr subtypes (>1 μ M), thus indicating that TTXs subtypes must play a critical role in the generation of ectopic discharges. Furthermore, a similar dose of TTX (12.5-50 nM) applied onto the ipsilateral L5 DRG in SNL rats was effective in reducing neuropathic pain behaviors (Lyu et al., 2000), thus supporting the hypothesis that TTXs subtypes are involved in neuropathic pain.

VI. NA_V1.3 VGSC MAY BE THE CRITICAL SUBTYPE FOR ECTOPIC DISCHARGE GENERATION

Once TTXs subtypes are identified as an important group for the generation of ectopic discharges, it is necessary to determine which of the six individual TTXs subtype VGSCs—Na_v1.1, Na_v1.2, Na_v1.3, Na_v1.6, Na_v1.7, and Na_x—mediate ectopic discharges. The responsible subtype was identified by examining the expression of TTXs subtypes that are upregulated



FIGURE 1 Comparison of changes of mRNAs for TTXs subtypes of sodium channels. The mRNAs of six TTXs subtypes ($Na_v1.1$, $Na_v1.2$, $Na_v1.3$, $Na_v1.6$, $Na_v1.7$, and Na_x) were measured in the DRG by an RNase protection assay at 1 day (1D) and 5 days (5D) after spinal nerve ligation. The amounts of mRNA are expressed as percentage of control (normal) values. The mRNA of four subtypes decreased after spinal nerve ligation, while two subtypes ($Na_v1.3$ and Na_x) increased. Upregulation of Na_x was evident only at postoperative day 5, while that of $Na_v1.3$ was obvious on both day 1 and 5. The data are adopted from Kim *et al.* (Kim *et al.*, 2001b, 2002).

in the DRG in synchrony with the development of ectopic discharges. The levels of mRNAs for the six TTXs subtypes were measured in the DRG with RNase protection assays (RPA) at various times after spinal nerve ligation (Kim et al., 2001b, 2002). The mRNAs of Na_v1.1, Na_v1.2, Na_v1.6, and Na_v1.7 declined from day 1 and afterward following the nerve ligation, while those of $Na_v 1.3$ and Na_v increased (Fig. 1). The increase of $Na_v 1.3$ mRNA was already evident at 16 h after spinal nerve ligation and then gradually climbed for up to 7 days. The increase of Na_x mRNA was, on the other hand, observed at 5 days but not at 1 day after the injury (Fig. 1). It has been shown that ectopic discharges develop 13 h after spinal nerve ligation and continue to be generated for a long period of time (weeks and months). Therefore, Na_v1.3 is the only subtype of TTXs VGSC whose mRNA is upregulated in the DRG in synchrony with the development of ectopic discharges. An immunohistochemical study of DRG neurons after spinal nerve ligation also showed that the number of Na_v1.3 immunoreactive DRG neurons was greatly increased, mostly in large-sized DRG neurons (Fig. 2) (Kim et al., 2001b). Assuming the upregulation of sodium channels is a critically important factor for the generation of ectopic discharges and neuropathic pain, these data suggest

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FIGURE 2 Photomicrographs of immunostained L5 DRGs for $Na_v1.3$ subtype of sodium channels. (A) and (B) show DRGs taken from the contralateral and ipsilateral sides, respectively, 1 day after unilateral tight ligation of the L5 spinal nerve. A much higher number of immunostained cells (mainly large diameter neurons) on the ligated side (some labeled neurons are indicated by arrow heads) is noted. Bar, 50 μ m.

that $Na_v 1.3$ is the important sodium channel subtype, although a possible role of the Na_x subtype at a later postoperative period cannot be ruled out. The results of our studies are consistent with others in that upregulation of the $Na_v 1.3$ sodium channel subtype has also been previously observed by others for a relatively long time after axotomy (7–9 days) (Waxman *et al.*, 1994; Boucher *et al.*, 2000).

VII. THE INVOLVEMENT OF TTXr VGSCs IN NEUROPATHIC PAIN

TTXr sodium currents are essential for action potential generation in the majority of nociceptive neurons under normal conditions (Phelps *et al.*, 1989; Ogata and Tatebayashi, 1993). The involvement of TTXr Na_v1.8 sodium channels in neuropathic pain is also evidenced by the observation that the hyperalgesia induced by spinal nerve ligation was reversed significantly when the transcription of Na_v1.8 channels was knocked down with antisense oligonucleotides (Lai *et al.*, 2002; Gold *et al.*, 2003). Because the expression of Na_v1.8 sodium channels and TTXr currents were significantly reduced in injured small DRG neurons (Dib-Hajj *et al.*, 1996; Cummins and Waxman, 1997; Dib-Hajj *et al.*, 1998), it is unlikely that Na_v1.8 action is

mediated by injured nerves. On the other hand, there is significant upregulation and redistribution of Nav1.8 channel expression (Novakovic et al., 1998) and enhanced TTXr current (Gold et al., 2003) in adjacent uninjured and sensitized neurons. Many uninjured C-fiber afferents also become spontaneously active (Wu et al., 2002) after nerve injury. It is likely that tissue injury, including nerve injury, results in local inflammation and the inflammatory mediators sensitize nociceptors and produce pain. Many inflammatory mediators and trophic factors are known to modulate the activity of TTXr sodium currents (Gold et al., 1996; Fjell et al., 1999; Cardenas et al., 2001; Zhou et al., 2002) and upregulate Nav1.8 in small DRG neurons (Bielefeldt et al., 2003). Thus, Nav1.8 sodium channels are likely involved in the generation of neuropathic pain after nerve injury through upregulation and redistribution of Na_v1.8 channels in uninjured nociceptive fibers. Therefore, it is plausible that both TTXs and TTXr subtypes play important but somewhat different roles in neuropathic pain. An injured peripheral nerve usually contains a mixture of axotomized and neighboring intact but inflamed afferent fibers. TTXr sodium channel subtypes may be involved in the generation of spontaneous activity in intact but sensitized nociceptors, whereas TTXs subtypes are involved in the development of ectopic discharges in axotomized afferents.

VIII. CONCLUSIONS

It has long been suggested that changes in sodium channels play an important role in ectopic discharge generation in injured afferent nerves and the subsequent development of neuropathic pain. Cloning and characterization of multiple subtypes of sodium channels have made it feasible to identify the subtype that is critically important. Studies indicate that both ectopic discharges and neuropathic pain are extremely sensitive to TTX, suggesting that TTXs subtypes are critically involved in these phenomena. Systematic examination of the mRNA of TTXs sodium channel subtypes in the DRGs of injured segments revealed that $Na_{y}1.3$ and Na_{x} are the two subtypes that are upregulated in the DRGs of axotomized segments, suggesting that they are potentially important subtypes in neuropathic conditions. Furthermore, upregulation of Nav1.3 was synchronous with the development of ectopic discharges, whereas that of Na_x was only apparent at a later point in time. These data suggest that Nav1.3 is the important sodium channel subtype for the generation of ectopic discharges and neuropathic pain after peripheral nerve injury. While TTXs subtypes are important for the generation of ectopic discharges in axotomized afferents, TTXr sodium channel subtypes may play an important role in sensitizing intact 10. Voltage-Gated Sodium Channels and Neuropathic Pain

(nonaxotomized) nociceptors, further contributing to neuropathic pain. Therefore, both TTXs and TTXr sodium channel subtypes seem to play important but somewhat different roles in neuropathic pain generation mechanisms.

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CHAPTER 11

Voltage-Gated Potassium Channels in Sensory Neurons

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I. OVERVIEW

The intrinsic electrical excitability of nociceptive neurons is an important component of the pain response. Nociceptor hyperexcitability associated with neuropathic pain is due to altered expression, localization, and/or activity of voltage-gated ion channels in the nociceptive membrane. Voltage-gated potassium or K_v channels are critical yet variable regulators of intrinsic excitability in mammalian neurons, and recent studies have revealed

changes in K_v currents and K_v channel subunit expression with treatments that lead to nociceptor hyperexcitability and neuropathic pain. Here, we review the literature on native K_v currents and K_v channel subunit expression in nociceptive neurons and how these change in response to nerve injury. We then discuss, in light of these findings, the potential of specific K_v channels as therapeutic targets for neuropathic pain.

II. INTRODUCTION

Individual neurons have characteristic and different intrinsic membrane properties that affect how they respond to various stimuli (e.g., synaptic, electrical, or environmental). A neuron's intrinsic excitability is a reflection of the expression patterns and localization of their voltage-gated ion channel repertoire (Hille, 2001). Importantly, a neuron's intrinsic membrane properties are not immutable, they can change during development and aging, subsequent to injury, in response to neuromodulatory signals from neighboring neurons or glial cells, in response to excitatory synaptic stimulation, and as a result of its inherent spontaneous electrical activity (Marder and Prinz, 2003). Voltage-gated K^+ or K_v channels are major determinants of a neuron's intrinsic excitability, due to their important roles in setting the resting membrane potential, the frequency, duration and amplitude of action potentials, and the extent and nature of forward and back-propagating action potentials (Hille, 2001). Moreover, the tremendous structural and functional diversity of K_v channels (MacKinnon, 2003), their highly variable patterns of expression and localization (Trimmer and Rhodes, 2004), and the extent of their dynamic modulation (Levitan, 1999) provide the principal basis for variability in the intrinsic membrane properties between different neurons and plasticity in response to specific physiological and pathophysiological conditions.

Nociceptors are a subpopulation of primary sensory neurons that are activated by "noxious" or painful stimuli. Sensory ganglia contain a variety of primary sensory neurons (Lawson, 1992). Among these are myelinated, fast-conducting, large-diameter fibers called $A\beta$ fibers, which have a low threshold for activation and mainly conduct proprioceptive and mechanoreceptive (e.g., touch) stimuli; the thinly myelinated medium-velocity fibers called $A\delta$ fibers, which are polymodal in nature; and the unmyelinated, slow-conducting small-diameter fibers called C-fibers, most of which have high threshold for activation and transmit painful stimuli. The characteristic conduction velocities of these different sensory fibers are due to a combination of diameter, myelination state, and ion channel expression (Harper and Lawson, 1985a). Among the population of nociceptive afferents, there are

unique subpopulations (Lawson, 1992), including afferents responsive to noxious thermal stimuli (i.e., C-cold fibers); noxious thermal and mechanical stimuli (i.e., C-mechano-heat fibers); and noxious thermal, mechanical, and chemical stimuli (C-polymodal). The sensitivity to different stimuli arises from their differential expression complement of different members of the Trp family of ligand-gated ion channels (Gunthorpe et al., 2002). However, a large body of evidence suggests that these cells may also express unique repertoires of voltage-gated ion channels that confer cell-specific properties of intrinsic excitability. Compelling evidence suggests that functional plasticity of nociceptors, due to short- and long-term modulation of receptor and ion channel activity, contributes substantially to the increased pain one feels in the presence of injury (Woolf and Salter, 2000). That many of these receptors and ion channels are found specifically in nociceptors makes these proteins attractive targets for eliminating pain without inducing side effects (McCleskey and Gold, 1999). Finally, the accessibility of the peripheral nervous system makes nociceptors a logical target for the development of modality specific therapeutics.

A diverse group of ligand- and voltage-gated ion channels transduce stimuli into depolarizations that are conducted along nociceptor axons and finally converted into neurotransmitter release. For example, nociceptive neurons express a variety of ligand-gated ion channels that are receptors for noxious stimuli such as temperature (TRPV1, TRPV2, TRPV3, TRPV4, TRPM8, and TRPA1) (Tominaga and Caterina, 2004), acid (ASICs1-4) (Krishtal, 2003), ATP-gated channels (P2X, P2Y) (Kennedy et al., 2003), and others. Moreover, voltage-gated Na⁺ (Na_v) channels (Na_v1.8, Na_v1.9) (Lai *et al.*, 2004) and voltage-gated $Ca^{2+}(Ca_y)$ channels (Ca_y2.2 and others) (Elmslie, 2004) represent attractive targets for therapeutic intervention of peripheral pain (Birch et al., 2004). Voltage-gated cation channels are key determinants of neuronal signaling through control of membrane excitability. Nav and Cav mediate inward cation fluxes that depolarize the nociceptive membrane and act as transducers of nociceptive signals. Conversely, K_v channels mediate fluxes that hyperpolarize or repolarize the nociceptive membrane and act as modulators and suppressors of nociceptive neuronal excitability. While the K_y channel subunits found in nociceptors to date are not unique to the nociceptive membrane per se, their key role in regulating nociceptor function, that they are dramatically regulated in neuropathic pain models, and the fact that certain K_{y} subunit combinations may be uniquely used in nociceptive membranes makes them attractive therapeutic targets for pain therapy (Wood et al., 2004). This chapter will focus on these K_v channels, which are critical to regulating nociceptor excitability; other chapters here deal with Na_v and Ca_v channels in the nociceptive membrane.

III. NOCICEPTOR EXCITABILITY

Sensory ganglia, such as the dorsal root ganglion (DRG), trigeminal ganglion, and others contain different and distinct populations of sensory neurons (Lawson, 1992). These neurons comprise a diverse population that can be classified based on morphological, functional, and molecular criteria. For example, DRG neurons with large somata are those that give rise to myelinated A α and A β fibers, which conduct tactile and proprioceptive information at high velocities. Smaller DRG somata give rise to $A\delta$ and C-fibers that conduct information from pain and thermal receptors at slower velocities. Small-diameter sensory neurons that are primarily nociceptors can be further divided neurochemically into two populations—isolectin B4 (IB4)-positive nonpeptidergic neurons and IB4-negative peptidergic neurons. It has been shown that IB4-positive neurons depend on glial-derived neurotrophic factor (GDNF) for survival, whereas IB4-negative neurons depend on NGF for survival during postnatal development (Molliver et al., 1997). Furthermore, these two populations of nociceptors terminate in distinct regions of the superficial spinal cord. IB4-negative cells project to lamina I and outer lamina II, whereas IB4-positive neurons terminate predominantly in inner lamina II (Silverman and Kruger, 1990). Studies have shown that IB4-positive neurons have longer-duration action potentials, higher densities of TTX-resistant sodium currents, and smaller noxious heat-activated currents than IB4-negative neurons. The different electrophysiological properties expressed by IB4-positive and -negative small neurons (Stucky and Lewin, 1999) indicate that they may relay distinct aspects of noxious stimuli both acutely and after injury in vivo.

IV. BASICS OF K_V CHANNELS

The mammalian K_v gene family contains approximately 20 gene encoding groups of highly related α subunit polypeptides. Each α subunit has a core domain with six transmembrane segments, S1–S6, and long cytoplasmic amino and carboxyl-termini. The highly conserved core domain contains the voltage sensing and pore-forming functionality of the K_v channel as well as all of the extracellular domains. The cytoplasmic amino and carboxyl terminal segments are less well conserved among K_v channel α subunits and are involved in protein–protein interactions with one another, with portions of the core domain, with auxiliary subunits, and with a diverse group of channel interacting proteins. Four α subunits assemble to form a functional K_v channel.

The K_v channel gene family has four branches, named $K_v 1-K_v 4$. K_v channel α subunits from one branch (e.g., $K_v 1$) can form heteromeric channels with one another but not with members of the other branches.

The K_v1 branch is the most extensive, with six members (K_v1.1–K_v1.7) expressed in the nervous system. These different mammalian K_v1 family members have distinct functional properties when expressed alone (i.e., as homotetramers) in heterologous cells. For example, K_v1.4 generates transient currents similar to the I_A in certain neurons, while other K_v1 family members generate sustained I_K -like K_v currents. Different K_v1 family members can also coassemble into channels with mixed subunit composition, and such heterotetrameric channels exhibit functional properties intermediate between those of channels formed from homotetramers of the constituent subunits.

The bulk of $K_v 1$ channel complexes in mammalian brain have associated $K_v\beta$ subunits (Rhodes *et al.*, 1997). Four K_v subunit genes exist in the human genome, and alternative splicing can generate a number of functionally distinct isoforms. Inclusion of the $K_v\beta 1.1$ subunit in K_v channel complexes containing $K_v 1.1$ or $K_v 1.2$ dramatically alters the channel-gating properties, converting the channels from sustained or delayed-rectifier I_{K} -type to rapidly inactivating or I_A -type. The specific α and β subunit composition of native complexes can dramatically impact both the expression level and function of $K_v 1$ channels in mammalian neurons. Moreover, heteromeric assembly with one another and coassembly with auxiliary K_v subunits can generate a diversity of function from the resultant $\alpha_4\beta_4$ channel complexes. The predominant $K_v 1$ cellular staining pattern throughout the brain is neuronal and subcellularly axonal.

 K_v2 family members $K_v2.1$ and $K_v2.2$ form delayed rectifier I_K -like K_v channels that are prominently expressed in mammalian brain, where they are localized in the somatodendritic domain of neurons. K_v3 family members ($K_v3.1-K_v3.4$) have unique functional characteristics, including fast activation at voltages positive to 10 mV and very fast deactivation rates. These properties are thought to facilitate sustained high-frequency firing, and K_v3 subunits are highly expressed in fast-spiking neurons such as neocortical and hippocampal interneurons. The $K_v4 \alpha$ subunits, $K_v4.1$, $K_v4.2$, and $K_v4.3$, form transient or A-type K_v channels. Immunoreactivity for K_v4 subunits is concentrated primarily in the dendrites of central neurons. Much progress has been made toward defining the cellular and subcellular distribution, subunit composition, and functional importance of different K_v channel subunits in mammalian brain. Much less is known of their specific contributions to the function of nociceptive neurons.

V. CLASSES OF NATIVE K_V CURRENTS

Native K_v currents in mammalian neurons have been divided into a large number of subtypes depending on their biophysical and pharmacological characteristics and their sensitivity to modulation (Pongs, 1999). The

biophysical properties of K_v channels determine the membrane potentials at which they will be available for opening, whether and how quickly they open in response to particular changes in membrane potential, the duration for which they remain open, and the rate at which they flux K^+ across the membrane (MacKinnon, 2003). K_v channels as a group vary much more extensively in these biophysical properties than other voltage-gated channels (Jan and Jan, 1997). While these inherent differences in biophysical properties are clearly encoded within the subunit composition of the channel complex and the primary structure of the particular component subunits, they can also be modified through dynamic covalent modification, usually phosphorylation (Levitan, 1999), and noncovalent protein-protein interactions (Jonas and Kaczmarek, 1996; Yi et al., 2001). The biophysical characteristics can also be dramatically modified pharmacologically, a fact that serves as the basis for a many promising therapeutics (Wickenden, 2002). However, specific modulators for many K_v channel subtypes have not yet been identified.

 K_{v} channels have been divided into a number of broad classes based on their biophysical characteristics (Hille, 2001). These characteristics define the role of the corresponding K_v current in the cell's physiology, as they determine the amount of active K_{y} current that flows under a given physiological condition. The first critical characteristic is the threshold and slope of the voltage dependence of channel activation. K_v channels can be divided roughly into those with low thresholds, which activate in response to relatively small depolarizations from the resting potential, or high thresholds, which require large depolarizations to transit from the closed to open states. K_{v} channels can also be classified based on the kinetics of their activation (rapidly or slowly activating). K_v channels can also inactivate, with characteristic kinetics (rapidly, slowly or noninactivating), in the face of a sustained depolarization. Inactivated channels are usually not available for reopening unless exposed to a repolarized (resting) membrane potential for some period, the kinetics of this recovery from inactivation are also characteristic of a given K_{v} channel. Lastly, K_{v} channels can differ dramatically in their conductance, ranging from high-conductance channels ($\approx 200 \text{ pS} = 10^8 \text{ K}^+$ ions/s) to those that have relatively low conductance ($\approx 2 \text{ pS} = 10^6 \text{ K}^+ \text{ ions/s}$) (Hille, 2001).

We will use these biophysical characteristics to define below the major classes of native K_v channels in nociceptive neurons, although it is important to remember that these characteristics of K_v channel function define the impact that a particular K_v channel type will have on neuronal excitability. However, while these somewhat subjective terms (low versus high threshold, inactivating versus noninactivating, high versus low conductance) can be quite useful as a starting point for describing K_v channel characteristics, in reality native K_v channels exhibit a tremendous diversity of function

(Jan and Jan, 1990). Moreover, K_v channel function is highly plastic and can be dynamically modified by phosphorylation (Levitan, 1999). In some cases, one can almost reach the conclusion that there is a dynamic continuum of function across the spectrum of native K_v channels. A typical experiment usually involves recording the whole cell K_v currents, representing the combined response of large numbers of functionally overlapping channels to a given stimulus. As such, precise definitions of native K_v current types based on their biophysical characteristics are sometimes difficult.

Moreover, pharmacological definition of K_v channels has been limited by a relative lack of naturally occurring neurotoxins and other functional modulators specific for individual K_v channel subtypes and by a dearth of selective therapeutic agents that target specific K_v channels (Wickenden, 2002). However, a library of somewhat selective K_v channel modulators is available, and the use of various combinations of these albeit imperfect blockers can lead to a useful pharmacological definition of a given K_v channel. Taken together, the biophysical attributes of a given channel can help to define what role it may be playing in nociceptor excitability, and pharmacological approaches do allow for direct experimental intervention in function that can help define the role of a given pharmacological class of channels to cell physiology. However, the lack of precise pharmacological tools can oftentimes lead to less than optimal pharmacological dissection and confusion in the literature. As molecular determinants of native K_y currents in nociceptive neurons are defined (see the following section), there is hope that specific genetic intervention experiments may lead to more decisive experiments as to the specific role of a given K_v channel in a given aspect of nociceptive signaling. However, studies utilizing more classical electrophysiological and pharmacological approaches have defined a number of native K_v currents that serve as candidates for therapeutics aimed at modulating nociceptor function and pain.

VI. NATIVE K_V CHANNELS IN NOCICEPTIVE NEURONS

Most studies on the native K_v channel repertoire of nociceptive neurons have come from electrophysiological analyses of DRG neurons. These studies have for the most part been performed recording from the DRG cell body. Under normal conditions, it is generally assumed that the DRG cell body is not a participant in sensory processing. DRG neurons are pseudobipolar, and impulse activity from the periphery to central targets does not require spike activity in the DRG somata. However, the complement of ion channels on the DRG cell body is thought to be representative of channels that are functionally important on the axon. Moreover, DRG neuronal cell bodies can become a source of ectopic impulse generation and contribute to the pathophysiology following nerve injury (Devor, 1994). Therefore, an understanding of K_v currents in DRG neurons, as determined by recordings from the cell body, is relevant to our understanding of nociceptor signal transduction.

Analyses of DRG K_v channels have been performed in a number of different experimental animals, and on DRG slices, on acutely isolated individual DRG neurons, and on cultured DRG neurons. This can infuse complexity into interpretation of data from different studies, as each experimental system can have characteristics distinct from the others. Moreover, the species and age of the animal used as the source of the study sample, and for cultured DRGs, the time and condition of the culture itself, can have profound impact on the results obtained. As such, while these studies have revealed certain commonalities, there exist differences in specific aspects of the biophysical and pharmacological properties of the expressed currents. Dissection of specific components of the total K_v current in most neurons is complex. Many subtypes with overlapping properties exist, and these properties can be modulated by assembly with other channel forming subunits or with auxiliary subunits and by phosphorylation (Jan and Jan, 1990). Moreover, inherent differences in the different preparations that have been studied (species, age of animal, method of preparation, recording conditions, and so on) make direct comparisons of the results of different studies difficult. However, it is clear that nociceptive neurons express multiple K_v currents, and molecular studies described later show complex repertoires of K_v channel gene expression. While a thorough review of each of the studies in an attempt to discern a unifying principle integrating all of the data from these studies is beyond the scope of this review, the functional importance of K_{v} currents and their attractiveness as therapeutic targets makes understanding the general aspects of these data worth considering. Moreover, such an analysis provides a useful foundation for interpreting data from studies performed on DRG neurons after treatments (axotomy, axonal constriction, and so on) known to induce hyperalgesia, in order to better understand the contribution of K_{v} channels to associated changes in nociceptor excitability. As such we will focus on the broad channel types defined in the studies on native K_v currents and then attempt to draw correlates to their molecular components.

For the purposes of this review, we will roughly divide K_v currents in DRG neurons into three major subclasses—the low threshold rapidly inactivating A-type or transient K_v current I_A , the high threshold noninactivating delayed rectifier K_v current I_K , and the low threshold, slowly inactivating K_v current I_D . The I_A can be further subdivided into fast (I_{AF}) and slow (I_{AS}) components. DRG neurons express variable combinations of these K_v currents, which also exhibit cell-specific differences in details of biophysical and pharmacological properties. As such, using such a broad classification scheme is a tremendous oversimplification of the diversity of K_v currents described in detailed and careful studies described in the literature. However, it serves as a useful starting point for discussions of the molecular components of nociceptive K_v currents below.

DRG neurons comprise a diverse population that can be classified based on both morphological and functional criteria. DRG neurons with large somata are those that give rise to A α and A β fibers, which conduct tactile and proprioceptive information at high velocities. Smaller DRG somata give rise to $A\delta$ and C-fibers that conduct information from pain and thermal receptors at slow velocity. The conduction speeds of these fibers are thought to arise from differences in axon diameter, myelination state and ion channel expression (Harper and Lawson, 1985a). However, studies on neurons within the DRG of anesthetized rats revealed an additional correlation between cell size and action potential duration, with small-diameter C-type cells having long action potentials (Harper and Lawson, 1985b). These intrinsic differences in excitability would be expected to be due to differences in the expression of voltage-gated ion channels in different populations of DRG neurons. We will mainly focus on studies in small diameter, low-conduction velocity sensory neurons, as these are the cells most likely to represent nociceptive neurons.

Stansfeld and Wallis (1985) provided some of the first evidence that different sized sensory neurons, in this case in rabbit nodose ganglia, expressed different complements of K_v currents. Small C-type cells were found to have a prominent low threshold, rapidly inactivating I_A , while larger diameter A-type cells had a more prominent high threshold, slowly or noninactivating $I_{\rm K}$. The presence of these distinct K_v currents correlated with the different firing properties in these two cell populations. Campbell (1992) provided further evidence, in this case from frog DRG, that different sized neurons expressed different complements of K_v channels. In addition to two species of Na_v channels, he found two different non- or slowly inactivating delayed rectifier type $I_{\rm K}$ components that exhibited differential distributions in large- and small-diameter frog DRG neurons. The $I_{\rm K}$ components could be easily distinguished by their activation kinetics. The largediameter cells, which had a rapidly activating and inactivating Nav current sensitive to the neurotoxin tetrodotoxin, also expressed a rapidly activating $I_{\rm K}$ component. This combination of channels with rapid kinetics led to short-duration action potentials in large-diameter cells, consistent with the properties of somata associated with mammalian A α and A β fibers. Smalldiameter cells had a slowly activating and inactivating, tetrodotoxinresistant Na_v current and a more slowly activating $I_{\rm K}$. These cells exhibited

slower action potential kinetics more typical of mammalian DRG somata that give rise to nociceptive $A\delta$ and C-fibers. Transient or A-type currents (I_A) were not apparent in the published recordings. This study paved the way for later studies defining distinct Na_v subtypes in nociceptive neurons (Lai *et al.*, 2004) as well as further studies on differential expression of K_v channel subtypes in different DRG cell types. A number of subsequent studies (e.g., McFarlane and Cooper, 1991; Gold *et al.*, 1996; Everill *et al.*, 1998; and many others) revealed further heterogeneity in both the I_A and I_K components as described in the following text.

Gold et al. (1996) performed a detailed biophysical and pharmacological characterization of K_v currents in DRG neurons cultured from adult rats. These studies revealed at least six distinct K_v currents, of which four exhibited differential distribution among different sized populations of DRG neurons. The predominant I_A was referred to as I_{AHT} , due to its transient nature and high threshold for activation. An I_{AS} , slowly inactivating A current was also observed. Both of these currents were preferentially expressed in small diameter, presumably nociceptive cells, as opposed to larger-diameter proprioceptive and mechanosensitive cells. That these I_{AHT} and I_{AS} expressing cells were nociceptive was underscored by the fact that they exhibited robust capsaicin responses. I_{AHT} was quite sensitive to blockade by 4-AP and TEA, while I_{AS} was more resistant to 4-AP and insensitive to TEA. This pharmacology, as well as the biophysical characteristics of these channels, suggests that I_{AHT} and I_{AS} may be encoded by $K_v 1.4$ and $K_v 4$ family α subunits, respectively (see later). These studies also revealed a set of $I_{\rm K}$ or delayed rectifier currents that also exhibited a cellular distribution that correlated with cell size. One of these delayed rectifier currents, IKi, which exhibited pronounced steady-state inactivation, was also preferentially expressed in presumptive nociceptive neurons, while I_{Kn} was found preferentially in larger diameter, nonnociceptive cells. At least some component of these currents may correspond to channels containing $K_v 1.1$ and/or $K_v 1.2 \alpha$ subunits (see later).

Everill *et al.* (1998) undertook a similar detailed study of K_v currents in acutely dissociated adult rat larger diameter (39–49 µm diameter) identified cutaneous DRG neurons. They found three major currents, which they termed I_A , I_K , and I_D . Different cells had differing levels of these three currents, which could be isolated using biophysical and pharmacological dissection. I_A was found in $\approx 80\%$ of the cells examined and exhibited characteristics most similar to the I_{AF} current found by Gold *et al.* (1996) in small-diameter nociceptive cells. I_K was most similar to the I_{Klt} of Gold *et al.* (1996) in that it had a fairly low threshold for activation. I_D corresponded closely to the I_{AHT} current of Gold *et al.* (1996), also termed I_{AS} by others.

Rola *et al.* (2003) identified similar K_v currents in cardiac DRG neurons. They focused their studies on small (<30 µm) and medium (31–48 µm) diameter cells and found no obvious differences in K_v currents present in the two morphological classes of cells. Their study focused on three K_v currents, I_{AF} (similar to the I_{AF} of Gold *et al.* (1996) and I_A of Everill *et al.* (1998)), I_{AS} (similar to the I_{AS} of Gold *et al.* (1996)), and I_K (similar to I_{Kn} of Gold *et al.* (1996)). I_{AF} and I_{AS} also exhibited pronounced differences in their slow and fast time, respectively, to recover from inactivation. This characteristic distinguishes $K_v 1.4$ -based I_A (slow recovery) from $K_v 4$ -based I_A (fast recovery). However, as heteromeric assembly of $K_v 1$ and $K_v 4$ family α subunits with one another and with auxiliary subunits can affect the biophysical properties of these channels, care should be exercised in assigning molecular definitions to native channels based on biophysical properties alone.

These currents have been further dissected pharmacologically using K_{y} specific neurotoxins from venomous snakes. The dendrotoxin (DTX) family of peptide neurotoxins, from mamba snakes, has been especially useful in this regard. DTX homologs exhibit high affinity binding and block of channels formed of certain α subunits within the K_v1 family. α -DTX from the green mamba Dendroaspis angusticeps blocks Ky1.1-, Ky1.2-, and Ky1.6-containing K_v channels (Stuhmer *et al.*, 1989; Akhtar *et al.*, 2002). DTX_K from the black mamba Dendroaspis polylepis polylepis exhibits high affinity block of channels containing one or more K_v1.1 subunits (Imredy and MacKinnon, 2000; Akhtar *et al.*, 2002). In DRG neurons, α -DTX exhibits a specific block of sustained $I_{\rm K}$ and induces hyperexcitability (Stansfeld and Feltz, 1988). α -DTX does not affect the fast (I_{AF}) component of I_A but blocks the slowly inactivating I_{AS} (Penner et al., 1986; Stansfeld and Feltz, 1988; Hall et al., 1994; Everill et al., 1998). The slowly inactivating IAS is also blocked by DTX_K , suggesting that the channels underlying this K_v current contain $K_v 1.1$ (Beekwilder *et al.*, 2003). That DTX_K did not block the α -DTX-sensitive noninactivating $I_{\rm K}$ (Beekwilder *et al.*, 2003) suggests that the K_v channels responsible for this current contain $K_v 1.2$ and/or $K_v 1.6$ in the absence of $K_v 1.1$. These data suggest that different $K_v 1 \alpha$ subunits in different heteromeric assemblies may underlie a major portion of the total I_{AS} and $I_{\rm K}$ current in DRG neurons. Moreover, as DTX block of these K_v channels induce nociceptor hyperexcitability, agents that increase the activity of these DTX-sensitive $K_v 1$ channels in DRG neurons are of potential therapeutic value.

VII. CHANGES IN K_V CURRENTS AND PAIN

Given the prominent role of K_v currents in regulating nociceptor excitability and that block of these currents induces hyperexcitability, it seems possible that suppression of K_v current expression and/or activity may underlie the nociceptor hyperexcitability associated with neuropathic pain or "pain initiated or caused by a primary lesion or dysfunction in the nervous system" (Suzuki and Dickenson, 2000). Neuropathic pain is characterized by sensations such as deep aching, increased sensitivity to noxious stimuli (hyperalgesia), and the perception of pain in response to innocuous stimuli (allodynia) (Zimmermann, 2001). Spontaneous and/or evoked hyperexcitability of sensory neurons is thought to contribute to these symptoms (Study and Kral, 1996; Zhang *et al.*, 1997; Zimmermann, 2001; Abdulla and Smith, 2001b; Ji and Strichartz, 2004). A number of experimental animal models have been utilized to determine how K_v currents may contribute to hyperalgesia and allodynia associated with nerve injury. Accumulating evidence indicates that altered expression of a number of voltage-gated ion channels, including K_v channels, are crucial in the injury-induced development of neuropathic pain due to sensory afferent hyperexcitability.

Studies comparing quiescent neuromas to those with spontaneous activity first suggested that altered levels of K_v currents could underlie hyperexcitability of sensory neurons (Devor, 1983, 1994). Everill and Kocsis (1999) first investigated the affects of axotomy on K_{y} currents in sensory neurons. These studies focused on the medium sized (46-50 µm diameter) cutaneous afferent DRG neurons that were the subject of their previous detailed biophysical and pharmacological K_v current characterization (Everill et al., 1998). Acutely dissociated L4 and L5 DRG neurons were isolated from adult rats 14–21 days after sciatic nerve axotomy. They found total peak current was reduced by 52% in the axotomized DRG neurons when compared with control cells. Using the classification $(I_{\rm K}, I_{\rm A}, I_{\rm D})$ developed in their previous study, they found that while I_D was unaffected by acute ligation, I_A was reduced by $\approx 60\%$ and I_K by >65%, compared with control cells. These results suggest a selective regulation of specific K_v currents in axotomized cells that may be related to the hyperexcitability of injured DRG neurons.

Abdulla and Smith (2001b) observed dramatic changes in all types of DRG neurons in axotomized rats, including increased excitability within 2–7 week of axotomy. Effects of axotomy were greatest in the small, putative nociceptive cells, moderate in the medium, and least in the large cells. Compared to control neurons, axotomized neurons exhibited a higher frequency of evoked action potential firing in response to depolarizing current injections, and the minimum current required to discharge an action potential was reduced. There were significant increases in spike duration and amplitude in small, presumably nociceptive cells from axotomized versus control animals. In the associated study, axotomy was found to affect a number of ionic conductances in all types (sizes) of DRG neurons examined (Abdulla and Smith, 2001a). $I_{\rm K}$ was the prominent K_v current in recordings

11. K_v Channels in Nociceptors

from both control and axotomized cells, and exhibited cell size-dependent reductions in amplitude (small $\approx 60\%$, medium $\approx 40\%$, large $\approx 20\%$). The smaller $I_{\rm AS}$ current was not affected. Comparison of current clamp data from Abdulla and Smith (2001b), and these voltage clamp data led to the conclusion that changes in $I_{\rm K}$ in themselves could be sufficient to explain the changes in excitability induced by axotomy.

A study focused on changes in currents in small-diameter DRG neurons after axotomy (Yang *et al.*, 2004). These studies found $I_{\rm K}$ and $I_{\rm AS}$ were expressed in small diameter presumably nociceptive neurons in DRGs from control rats. Axotomy led to large (50–60%) decreases in the amplitude of both $I_{\rm K}$ and $I_{\rm AS}$, suggesting that nociceptive neurons respond to nerve damage with changes in multiple $K_{\rm v}$ currents.

VIII. K_v Channel Gene Expression in Sensory Neurons

Neuronal function depends critically on both the expression level of ion channels in the plasma membrane, their subcellular localization (e.g., axon terminal, dendrite, soma, and so on), and their activity. As described earlier, there is a wealth of experimental data describing diverse K_v currents recorded from sensory neuron somata, as many important sites of action (e.g., nerve endings) are not accessible for electrophysiological analyses. While direct extrapolations from such data to the K_{v} currents that are important in normal or pathological sensory neuron function in vivo are not completely straightforward, they can be extremely informative as to the types of K_{y} currents found in nociceptive neurons and how these change in response to nerve injury. Moreover, there is compelling data that ectopic excitability of sensory neuron somata is an important consequence of treatments leading to neuropathic pain. However, combining other methods with these classical electrophysiological techniques are required to fully resolve the molecular identities and functions of K_v channels that underlie excitability of sensory neurons.

Several methods have now been employed to accomplish this goal. The first set of techniques is based on analyses of expression levels of K_v channel genes. Shortly after the isolation of the first mammalian K_v channel cDNAs, Northern blot analyses of K_v1 family mRNA levels in rat DRGs showed the presence of high levels of $K_v1.1$ message (Beckh and Pongs, 1990). Lower levels of $K_v1.2$ were also found in DRG, while $K_v1.3$ and $K_v1.4$ were present at barely detectable levels. Yang *et al.* (2004) examined the steady-state levels of K_v1 subunit mRNAs in samples isolated from freshly dissected DRG using sensitive and quantitative RNAse protection assays. They found robust expression of $K_v1.4$, $K_v1.4$

and K_v1.6 in control animals, consistent with the previous Northern blot study (Beckh and Pongs, 1990). They then extended these results by applying the same methods to examine $K_v l$ mRNA levels in DRG isolated from control and axotomized rats. They found dramatic decreases in steady-state mRNA levels for $K_v 1.4$ ($\approx 80\%$), $K_v 1.2$ ($\approx 60\%$), $K_v 1.3$ ($\approx 50\%$), and $K_v 1.1$ $(\approx 40\%)$. However, correlations between these axotomy-induced changes in K_v family mRNAs and those observed for K_v currents in this and other studies were not straightforward. One model is that the decrease in K_y 1.1 and $K_v 1.2$ underlies the observed decreases in the levels of DTX_K -sensitive $I_{\rm K}$. The reduction in $I_{\rm AS}$ seen here, but not in other studies, could be due to loss of K_v1.4- and K_v1.2-containing heteromeric channels or channels containing $K_v 1.2$ with inactivation-promoting $K_v \beta 1.1$ subunits (Rettig *et al.*, 1994). Alternatively, axotomy-induced changes in K_v currents could be due to posttranslational changes in K_vl channel trafficking, turnover or activity, or changes in expression of K_v channel genes outside of the K_v 1 family, neither of which would be detected in these studies.

An independent approach led to results similar to those obtained by Yang et al. (2004). An unbiased differential display analysis was used to identify any DRG gene whose expression was altered in response to chronic constriction injury of the sciatic nerve (Kim et al., 2001). This analysis turned up $K_v 1.2$ as an mRNA, among others, whose steady-state level was significantly reduced in axotomized DRG versus control. A subsequent report from the same group extended these studies by using quantitative RT-PCR approaches to investigate levels of each K_v family mRNA (Kim *et al.*, 2002). They found prominent decreases in expression of $K_v 1.1$, $K_v 1.2$, and $K_v 1.4$, consistent with the results described earlier (Yang *et al.*, 2004). Importantly, they also found marked reduction in expression of mRNA for the $I_{\rm K}$ -type K_v2.2 channel and for the $I_{\rm A}$ -type K_v4.2 and K_v4.3 mRNAs (Kim et al., 2002). These results were the first to identify K_v4 channel mRNAs in DRG. K_v4 family members are important components of I_A in mammalian central neurons (Birnbaum et al., 2004). The biophysical characteristics of IA in central neurons vary among neuronal types, depending on cell-specific interactions with auxiliary subunits and cell-specific K_v4 phosphorylation (Jerng et al., 2004). It is likely that the multiple components of I_A observed in studies of DRG neurons, some of which are described earlier, are based on K_v4 channels of variable auxiliary subunit composition and phosphorylation.

It should be noted that these batch analysis methods of expression level, which involve homogenization and mRNA extraction from whole DRG, do not allow for insights into which cells express the mRNAs in the absence of parallel *in situ* hybridization studies. Thus, a low level of expression in control animals (e.g., K_v 1.4) may reflect a low and uniform expression level

across many cell types (i.e., nociceptive and nonnociceptive neurons) or high-level expression in a small subset of cells (see later). The same holds for these batch analyses of changes upon axotomy. As an example, the 40% decrease in $K_v 1.1$ mRNA levels may reflect the complete disappearance of $K_v 1.1$ mRNA in a subset of cells or a partial and uniform reduction across all expressing cells. Moreover, steady-state mRNA expression levels are not necessarily reflected in steady-state protein expression levels, as demonstrated in a global analysis of mRNA and protein expression in a homogeneous population of yeast cells (Ghaemmaghami *et al.*, 2003).

IX. ANALYSES OF K_V CHANNEL PROTEIN EXPRESSION IN NOCICEPTIVE NEURONS

Given the considerations in an earlier section, strategies incorporating analyses of K_v channel protein expression levels across the entire DRG and in individual DRG neurons seem warranted. One approach is to employ antibodies directed against unique epitopes found in each K_{y} channel subunit in immunocytochemical and biochemical analyses of DRG. For example, an antibody directed against K_v1.1 and that does not recognize any other K_{v} channel subunit can reveal the population of sensory neurons that expresses this protein, the expression level relative to other neurons, and wherein the neuron the protein is found. Such information can provide important insights into the potential contribution of this channel subunit to the K_v currents important in nociceptor function. Furthermore, such K_v channel-specific antibodies can be used in conjunction with antibodies against other K_v channel subunits in multiple labeling experiments designed to correlate expression of K_v channel subunits with one another. As K_v channels are large multisubunit protein complexes of four subunits and up to four auxiliary subunits (Kim et al., 2004), the extent of colocalization of different subunits may reflect formation of heteromultimeric channels with biophysical and cell biological properties distinct from homomultimeric channels (see in an earlier section). K_y channel antibodies can also be used in multiple-labeling experiments in conjunction with immunocytochemical markers for functional populations of nociceptive neurons. For example, double immunostaining with antibodies against K_v1.1 and antibodies against the capsaic sensitive TRPV1 channel will reveal if K_y 1.1 is present in thermal nociceptors (Caterina et al., 1997).

We used this approach to identify $K_v l$ channel subunit proteins present in sensory neurons in the DRG before and after nerve injury. These experiments showed that sensory neurons of different functional modalities express unique channel subunits (Rasband *et al.*, 2001). Figure 1A shows DRG



FIGURE 1 $K_v l$ channel subtype expression in DRG. (A) Immunostaining of DRG for $K_v l.1-K_v l.6$ reveals that $K_v l.1$, $K_v l.2$, and $K_v l.4$ subunits are all present at high levels in DRG. Scale bar = 100 μ m. (B) Immunoblot analysis of brain and DRG membrane homogenates for $K_v l$ channel subunits, numbers to left of panel represent relative electrophoretic mobility of protein molecular weight standards, in kDa. Modified from Rasband *et al.* (2001).

immunostained using specific antibodies against $K_v 1.1-K_v 1.6$ (Rhodes *et al.*, 1995; Bekele-Arcuri *et al.*, 1996). These results show that although all $K_v 1$ mRNAs may be detected (Beckh and Pongs, 1990; Kim *et al.*, 2002; Yang *et al.*, 2004), only $K_v 1.1$, $K_v 1.2$, and $K_v 1.4$ subunit proteins can be detected. There are two obvious interpretations of these data—that the level of detection is such that these methods can only detect the most abundant

TABLE I

$K_v l$	Channel Subunits are	Widely Expressed in all DRG Neurons and form Heteromu	ltimeric
	Channels that	Give Rise to Sustained (I_K) or Transient (I_A) Currents	

K _v 1 channel subunit	Average dia- meter ^a (μm)	DRG locations	Subunit combinations ^b	Expected currents ^c
K _v 1.1	38 ± 9	Mechanoreceptor	K _v 1.1/K _v 1.2	Sustained
		Proprioceptor	$K_v 1.1/K_v 1.4K_v 1.1/K_v 1.2/K_v 1.4$	Transient
K _v 1.2	33 ± 11	Mechanoreceptor	$K_v 1.1/K_v 1.2 K_v 1.1/K_v 1.2/K_v 1.4$	Sustained
		Proprioceptor		Transient
K _v 1.4	26 ± 10	Nociceptor	K _v 1.4	Transient
		Mechanoreceptor	$K_v 1.1/K_v 1.4$	
		Proprioceptor	$K_v 1.1/K_v 1.2/K_v 1.4$	

The size of the text for DRG locations, subunit combinations, and expected currents reflects the predominant properties for each type of subunit. It is important to emphasize that other K_v channels (non- $K_v l \alpha$ subunit containing) are likely present in DRG neurons and contribute to the K^+ currents in these cells; the molecular composition of these channels remains unknown.

^aAverage diameters taken from Rasband et al. (2001).

^bSubunit combinations from Rasband et al. (2001) (includes cells of all diameters).

^{*c*}Any channel containing one or more $K_v I.4 \alpha$ subunits is expected to exhibit a transient current (I_A), and those without $K_v I.4$ subunits produce sustained currents (I_K).

proteins (K_v 1.1, K_v 1.2, and K_v 1.4 are probably the most abundant DRG K_v 1 channel mRNAs) or that there is a disconnect between K_v 1.3, K_v 1.5, and K_v 1.6 mRNA and protein. Immunoblot analyses of crude membrane fractions prepared from DRG were run in parallel to the immunofluorescence staining (Fig. 1B). In membranes isolated from DRG, K_v 1.1, K_v 1.2, K_v 1.4, and K_v 1.6 were detected in significant amounts. It should be noted that the lower molecular weight bands seen in the K_v 1.1, K_v 1.2, and K_v 1.4 immunoblots correspond to K_v 1 channel subunits with high mannose oligosaccharide chains (Shi and Trimmer, 1999; Manganas and Trimmer, 2000). These same K_v 1 channel subunits were also detected in sensory neurons by our immunofluorescence staining of trigeminal ganglia (MNR and JST, unpublished results), and a report using a different anti- K_v 1.2 antibody also showed robust immunofluorescence staining in trigeminal ganglia (Ichikawa and Sugimoto, 2003). Thus, the prominent K_v 1 subunits found expressed at the mRNA level are also found at the protein level in sensory neurons (Table I).

It is important to keep in mind that the results presented here represent only a subset of the K_v channel subunits expressed in the nervous system (Trimmer and Rhodes, 2004). As mRNA studies reveal prominent expression of K_v2 , K_v3 , and K_v4 family members in DRG (Kim *et al.*, 2002), detailed analyses of the expression levels and cellular and subcellular localization of the corresponding proteins are needed. Furthermore, channel function can be dramatically altered by the influence of auxiliary subunits (Trimmer, 1998). As discussed earlier, for example, it is now clear that other kinds of K_v channels and their accessory proteins (e.g., K_v4 and KChIPs, respectively) (An *et al.*, 2000) may be found in peripheral sensory neurons (MNR and JST, unpublished results). We anticipate that as new antibodies and reagents become available, investigators will arrive at a more complete picture of the kinds of K_v channels present in sensory neurons.

X. K_v1 CHANNEL SUBUNITS AND SUBUNIT COMBINATIONS DEFINE DISTINCT POPULATIONS OF SENSORY NEURONS

As mentioned earlier, to correlate the expression of a given channel subunit with a specific K_v current, it is important to determine the expression levels of different K_{y} channel subunits on a cell-by-cell basis. This is possible using immunofluorescence staining of sensory neurons in DRG sections. DRG neurons with different functional modalities have characteristic sizes, such that simple correlations can be drawn between K_v channel staining and cell size (Rasband et al., 2001). It is also possible to use a variety of markers to distinguish between functionally unique sensory neurons. For example, subsets of sensory neurons express unique molecular components including specific kinds of Na_v channels (e.g., Na_v1.8 and Na_v1.9), Trp ion channels, neurotrophin receptors (Trk receptors), neuropeptides (e.g., calcitonin generelated peptide [CGRP], substance P), surface carbohydrates, or neurofilament/intermediate filament proteins (e.g., peripherin, NF200) (Dib-Hajj et al., 1998; Novakovic et al., 1998; Amaya et al., 2000; Moran et al., 2004). Therefore, antibodies against these different proteins can be used in multiple-label immunostaining experiments to determine if specific K_v channel subunits are localized to specific functional groups of sensory neurons. As an example, an antibody (Rasband et al., 1999a) that recognizes all voltage-gated Na_v channel subtypes (pan-Na_v) (Fig. 2A) labels most intensely those DRG neurons that express Nav1.8 and Nav1.9 (Rasband et al., 2001 and MNR, unpublished results). These neurons are known to be C-type nociceptive neurons (Lai et al., 2004). Importantly, many of the sensory neurons that express high levels of the $K_v 1.4$ subunit are those most intensely labeled with pan-Na_v immunoreactivity (Fig. 2A and B, arrow). This result strongly suggests that $K_v 1.4$ is expressed in nociceptive C-fibers.

As mentioned earlier, another common method to determine functional modality is to measure cell diameter. Roughly, large- and medium-diameter neurons correspond to $A\alpha$ and $A\beta$ neurons with myelinated axons, while small-diameter neurons correspond to thinly myelinated $A\delta$ and C-fibers.

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FIGURE 2 K_v1 channel subunits define distinct populations of sensory neurons. (A) and (B) Pan-Na_v and K_v1.4 double-label immunofluorescence staining shows high-level expression of Na_v channels and K_v1.4 in common cells (arrow). (C), (E), and (G) Histograms showing the number of cells of a given diameter labeled using antibodies against K_v1.1 (C), K_v1.2 (E), or K_v1.4 (G). (E), (F), and (H) Triple-label immunofluorescence for K_v1.1 (D), K_v1.2 (F), and K_v1.4 (H). Scale bar = 100 µm. Modified from Rasband *et al.* (2001).

Different K_v1 channel subunits are detected in DRG neurons of various sizes: $K_v1.1$ is present in large-diameter neurons with an average size of $38 \pm 9 \ \mu m$ (Fig. 2C), $K_v1.2$ is found in both large- and medium-diameter neurons with an average size of $33 \pm 11 \ \mu m$ (Fig. 2E), and $K_v1.4$ is detected mainly in small-diameter neurons, although some larger-diameter cells can be detected (Fig. 1A arrow) with an average diameter of $26 \pm 10 \ \mu m$ (Fig. 2G) (Rasband *et al.*, 2001).

Since K_v1 channels can heteromultimerize promiscuously with dramatic effects on function and biology (see in an earlier section), it is important to correlate the coexpression of these channels within sensory neurons. The most direct method to do this is through multiple-label immunofluorescence staining (Trimmer and Rhodes, 2004). This is made possible by using antibodies from different species and/or mouse monoclonal antibodies of different isotypes (Rhodes et al., 1997, 2004). We have performed such multiple-label immunofluorescence staining in DRG neurons for $K_{y}1.1$, K_v1.2, and K_v1.4 (Fig. 2D, F, and H) (Rasband et al., 2001). These experiments revealed a wide variety of potential subunit combinations that could dramatically influence the biophysical and pharmacological characteristics of K_{y} currents present in DRG neurons. For example, we found subsets of neurons that expressed mainly K_v1.1 (Fig. 2D, F, and H, double arrows), neurons that expressed only $K_v 1.4$ (Fig. 2D, F, and H, arrow), neurons that expressed both $K_v 1.1$ and $K_v 1.2$ (Fig. 2D, F, and H, arrowhead), and neurons that expressed all three subunits (Fig. 2D, F, and H, asterisk). In general, small-diameter cell bodies associated with unmyelinated C-fibers have high levels of K_v1.4, but large- and medium-diameter cell bodies associated with myelinated A-fibers have $K_v 1.1$, $K_v 1.1/K_v 1.4$, $K_v 1.1/K_v 1.2$, or $K_v 1.1/K_v 1.2/K_v 1.4$ subunit combinations (Table I). The potential role of these and other K_v channel subunits in generating the native currents observed in nociceptive neurons is discussed below.

It should be emphasized here that staining of K_v channels, or any other plasma membrane ion channel or receptor, in the cell bodies of sensory neurons does not allow for direct conclusions as to the levels of that protein in functionally relevant domains of the cell. The ion channel and receptor antibody staining, others and we have obtained in DRG and other sensory ganglia, is specific for intracellular pools of these membrane proteins. These presumably represent recently synthesized populations of these proteins that are transiting through the rough endoplasmic reticulum (ER) and Golgi apparatus on their way to their ultimate plasma membrane sites of action and as such have no direct contribution to nociceptive function. That this staining is to an intracellular/biosynthetic pool is supported by a number of considerations. The first is that the nucleus of the cell is prominent as an immunoreactivity-negative "hole" in the center of sensory neuronal somata in all figures of ion channel/receptor staining published to date. This staining

pattern is distinct from what is obtained with many of these same antibodies in central neurons, where little intracellular accumulation of staining is observed (Trimmer and Rhodes, 2004). That the staining in DRG is to biosynthetic pools of channel proteins is supported by immunoblot data, as shown in Fig. 1. As mentioned earlier, these immunoblot reveal two different protein bands for K_v1.1, K_v1.2, and Kv1.4. Using K_v1.1 as an example, the DRG membrane sample has prominent bands at ≈ 97 kDa, and at ≈ 67 kDa, which represent K_v1.1 with complex or mature (Golgi, plasma membrane) and high mannose or immature (ER) asparagine-linked oligosaccharide chains, respectively. While the bands with the ER form of glycosylation are present in the membrane preparation from rat brain, the proportion of the mature to immature is quite different than in the DRG sample. These data show that membrane fractions prepared from DRG have high levels of K_v1.1, K_v1.2, and K_v1.4 channel protein with oligosaccharide chains typical of ER localized glycoproteins, consistent with the observed immunocytochemical staining pattern. It may be that the biosynthetic needs of populating the long processes of sensory neurons with sufficient quantities of channel proteins are such that relative to central neurons channel biosynthesis is maintained at very high steady-state levels in sensory neurons. In this scenario, levels of intracellular channel protein would be reflective of the functional pool of channels in the axonal membrane. Alternatively, the intracellular staining could be deceptive. Plasma membrane expression of ion channels is under strict posttranslational regulation (Ma and Jan, 2002; Misonou *et al.*, 2004). In the case of $K_v l$ channels, there exist strong trafficking determinants in extracellular segments near the channel pore that restrict trafficking to certain preferred subunit combinations (Manganas *et al.*, 2001), and K_y 4 channels have trafficking determinants in their amino termini (Shibata et al., 2003; Scannevin et al., 2004). This raises the specter of a very unattractive scenario, in that there exists an inverse correlation between the densities of channels "trapped" in the soma, and the density of channels functioning in the sensory processes. The same caveats hold for analyses of levels of ganglionic mRNA and of levels of ionic current in recordings from the soma. Given these considerations, it becomes important to begin to address the question of which channels are present at sites of action in the periphery, as we have begun to do for $K_v 1.4$.

XI. K_V CHANNEL LOCALIZATION IN SENSORY NEURONS

Consistent with their expression in large- and medium-diameter neurons, $A\alpha$ and $A\beta$ fibers have high levels of $K_v 1.1$ and $K_v 1.2$ channels present in zones immediately adjacent to nodes of Ranvier but restricted beneath the

myelin sheath in regions called juxtaparanodes (Wang et al., 1993). These channels are thought to modulate conduction properties during early development (Rasband et al., 1999b; Vabnick et al., 1999) and after injury (Rasband et al., 1998), but their function during normal action potential conduction is not well understood (Rasband, 2004). Some myelinated fibers also have $K_v 1.4$ at juxtaparanodes, but the functional significance of this differential expression is unknown (Rasband et al., 2001). Importantly, K_{y} 1.4 is present on unmyelinated axons both in the sciatic nerve and near nerve terminals. For example, in the bladder (a tissue rich in C-fibers) there are high levels of $K_v 1.4$ in unmyelinated axons. The staining for $K_v 1.4$ colocalizes precisely with immunostaining for VR1 (TRPV1) and CGRP (Rasband *et al.*, 2001). In the case of K_v 1.4, these channels appear to be present in a distribution along the axon that can modulate the properties of the conducted action potential (Rasband et al., 2001). Bladder nociceptive afferents have been reported to contain a K_v1.4-like rapidly inactivating A-type current (Yoshimura et al., 1996).

XII. K_v CHANNELS AND NERVE INJURY

Because neuronal function depends so critically on the expression levels of ion channels, one potential contributor to neuropathic pain after injury or disease is the altered expression of ion channels (Zimmermann, 2001). A major goal of pain research is to correlate the changes in nociceptive neuron excitability occurring after nerve injury with changes in the expression and/ or function of specific ion channels (Lai et al., 2004). As described earlier, a variety of changes in K_v currents occur after nerve injury. Since K_v channels are involved in modulating excitability either through setting the resting membrane potential or regulating action potential duration and frequency of firing, it is clear that loss of these channels could dramatically alter the firing properties of these neurons and lead to hyperexcitability. To address the changes in K_{y} channel protein expression associated with nerve injury. we combined the use of a well-established model of nerve injury (the Chung model [Kim and Chung, 1992]) and immunocytochemistry with Ky channelsubtype specific antibodies to investigate the consequences of nerve injury on K_vl channel expression and localization. As shown in Fig. 3, there is a dramatic reduction in the amount of all K_y channels assayed in DRG cell bodies after nerve injury. The observation that chronic bladder inflammation increases the excitability of nociceptors through a reduction in A-type K_v currents (Yoshimura and de Groat, 1999) is consistent with the reduction in K_v1.4 immunoreactivity seen in the injured small-diameter neurons. However, the data shown in Fig. 3 is restricted to an investigation of the



FIGURE 3 There is a dramatic reduction in the levels of $K_v l$ channel protein in DRG neurons from an injured nerve compared to control DRG. The injury was a tight ligation of the spinal nerve distal to L5/L6 DRG. Animals were sacrificed 7 days after injury. Contralateral DRG served as controls. Scale bar = 100 µm. Modified from Rasband *et al.* (2001).

neuron cell body. Thus, any interpretation of the functional significance of this downregulation must be tempered by the fact that the consequences for $K_v l$ channels in axons (both myelinated and unmyelinated) remain to be determined.

XIII. GENETIC INTERVENTION IN K_V CHANNEL EXPRESSION

Given the identification of candidate K_v channel subunits that may be important in controlling the excitability of nociceptive neurons in normal and neuropathic states, how does one go about directly testing their contribution to nociceptor functions? As described earlier, in the case of K_v1 channels we have available the mamba toxins. Strategic use of these toxins has allowed for an identification of $K_v1.1$ as a key subunit in the K_v channels

underlying $I_{\rm K}$ (Beekwilder *et al.*, 2003). However, such definitive pharmacology for virtually all other K_v channels expressed in sensory neurons (e.g., $K_{y}1.2$, $K_{y}1.4$, $K_{y}4$ family) is not available. Thus, alternative approaches must be taken. One approach is genetic ablation of the corresponding gene. This has been accomplished for mouse $K_v 1.1$ (Smart *et al.*, 1998). The $K_v 1.1$ knockout mice display hyperalgesia, consistent with an important role for these channels in nociception (Clark and Tempel, 1998). The K_v currents recorded from somata of wild-type and K_v1.1 knockout mice are strikingly similar (Jiang et al., 2003), suggesting either compensatory upregulation of another K_v subunit, a disconnect between somal recordings and the situation in the periphery, or that the hyperalgesic phenotype of the $K_v 1.1$ knockouts is due to a central effect. Thus, while it seems clear that, based on biophysical and pharmacological characteristics, analyses of mRNA and protein levels, and knockout mice, that K_v1.1 is important in sensory neuron function and nociception. Identification of other K_v subunits contributing to nociceptive function may rely on similar multidisciplinary approaches. An attractive approach that has gained recent prominence is the use of RNA interference to effectively and specifically knockdown expression of specific target proteins in neurons (Buckingham et al., 2004; Di Giovanni et al., 2004). Using this approach to acutely suppress expression of candidate K_{v} channels in nociceptive neurons may lead to identification of channel subunits involved in nociceptive function under normal and neuropathic conditions.

XIV. CONCLUSIONS

 K_{v} channels comprise a large and diverse set of ion channels. As described, there is a rich variety in the kinds of K_v channel subunits and currents that can be found in sensory neurons. As new antibodies and reagents (e.g., specific toxins or channel blockers) become available, the complexity of channels present in sensory neurons and nociceptors in particular will likely expand. However, the precise molecular identities of some of these K_{y} channels have now been identified, and these channels can be used as targets for the treatment of neuropathic pain. As described earlier, the $K_v 1.4 \alpha$ subunit is enriched in nociceptors. Among the K_v 1 subunits, K_v 1.4 is unique in that it elicits an A-type current and undergoes rapid N-type inactivation (i.e., peptide ball inactivation). Furthermore, after nerve injury both the channel and the current are reduced. Thus, K_v1.4 represents an attractive therapeutic target for the treatment of neuropathic pain. It is not difficult to imagine that a drug that enhances or prolongs the activity of $K_v 1.4$, perhaps acting as a "disinactivator" of rapid K_v1.4 inactivation (Zhang et al., 2004), could reduce nociceptor excitability. Alternatively, since there is some

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evidence that after certain forms of nerve injury neurons can switch receptor modality (Wallace *et al.*, 2003), other members of the K_v1 channel family may also be attractive targets for the treatment of neuropathic pain.

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CHAPTER 12

Two-Pore Domain Potassium Channels in Sensory Transduction

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- I. Overview
- II. Introduction
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I. OVERVIEW

Certain ion channels mediate sensory transduction by virtue of their sensitivity to various sensory stimuli. Studies suggest that members of the two-pore K^+ (K_{2P}) channel family could be involved in sensory transduction, particularly in nociception. K_{2P} channels, such as TREKs and TASKs are expressed in dorsal root ganglion (DRG) and other sensory neurons. TREKs and TASKs are active at rest and therefore contribute as background K^+ channels that help set and stabilize the resting cell membrane potential. In addition, TREKs and TASKs are sensitive to various chemical and physical factors including heat, membrane tension, acid, and lipids.

Receptor agonists coupled to Gq/11 proteins inhibit both TREKs and TASKs. Thus, activation of TREKs by nociceptive stimuli, such as heat and acid, may oppose the depolarizing influence produced by warm/hot sensing TRPV ion channels. On the other hand, inhibition of TREKs by cold may increase excitability and facilitate cold-sensing mediated by TRPM ion channels. Cell-swelling and lipid molecules produced during inflammation may activate TREKs and modulate synaptic transmission and pain sensation. Inhibition of TREKs and TASKs by receptor agonists may be involved in transmission of sensory information. The unique properties of TREKs and TASKs suggest that they would be involved in the sensory transduction processes. Study of the potential roles of K_{2P} channels in sensory transduction in various sensory neurons, and identification of the underlying signaling pathways are important topics for the future.

II. INTRODUCTION

Nociception occurs when intense mechanical, thermal, or chemical stimuli increase the excitability of neurons of the primary $A\delta$ and C-afferent fibers, and a train of action potentials is generated at the nerve terminals. Action potentials thus generated are carried to several regions of the brain via the ascending pain pathways in the spinal cord. In order to initiate electrical signals at the nerve terminal, a nociceptive stimulus needs to produce depolarization of the neurons. This can be accomplished by activation of Na⁺ channels, or nonselective cation channels, or inhibition of K⁺ channels. One or more of these ionic mechanisms are probably involved in increasing neuronal excitability, facilitating generation of action potentials, and producing nociception. Thus, block of Na⁺ and Ca²⁺ entry, and activation of K⁺ channels would be antinociceptive.

Several types of ion channels sensitive to mechanical, thermal, and chemical stimuli have been identified in mammalian tissues. They include voltage-gated Na⁺ channels, ATP-gated ion channels (P2X receptor), acid-sensing ion channels (ASICs) that include epithelial Na⁺ channel (ENaC) family, transient receptor potential (TRP) ion channels, and two-pore domain (K_{2P}) channels. Sensory neurons, such as DRG and trigeminal neurons, express all of these ion channels that mediate innocuous and noxious sensations produced by various stimuli. Until now, most of the study of sensory transduction has focused on Na⁺ and nonselective cation channels as a mechanism of increased excitability in sensory neurons. Studies suggest that certain receptor agonists can increase neuronal excitability by inhibition of background K⁺ current. K_{2P} channels (also known as two-pore or tandem-pore domain K⁺ channels) are now known to be background K⁺ channels in many cell types and are also known to be

targets of various chemical and physical stimuli. As members of the K_{2P} channel family are expressed in various sensory neurons, they may also contribute importantly to the sensory transduction and perception.

III. K_{2P} CHANNEL FAMILY

A. General Properties

The mammalian K⁺ channel family consists of three structurally distinct classes: the voltage-gated (K_v) , inwardly rectifying (K_{ir}) , and two-pore domain (K_{2P}) channels. The first two K_{2P} channels (ORKI and tandem-pore weak inwardly rectifying K⁺ channel 1 [TWIK-1]) were identified by expression cloning and searching the DNA database for sequences similar to already cloned K⁺ channels, respectively (Goldstein et al., 1996; Lesage et al., 1996a). Further analysis of the DNA database and homology screening of cDNA libraries led to cloning of many other K_{2P} channels. The distinguishing structural characteristics of the K_{2P} channel subunits are that two-pore forming domains and four transmembrane segments are present in each subunit. Each pore-forming domain has the characteristic K⁺ channel signature sequence (TIGYG) or similar conserved sequences that form the selectivity filter for K^+ (Doyle *et al.*, 1998). The extracellular segment between the first transmembrane segment and the first pore is usually very long and contains one or more glycosylation sites. The amino termini are always very short but the carboxy termini are usually long and contain sites for phosphorylation and other modulators. The K_{2P} channels are presumably homo- or heterodimers, as functional K⁺ channel possess four poreforming domains. At present, there are 16 members within the mammalian K_{2P} channel family that can be subclassified into six related protein families (Fig. 1). Most K_{2P} channels form functional K^+ channels when expressed in oocytes and mammalian cloned cell lines. They behave as background (or leak) K^+ channels, as they show little or no time- and voltage-dependence within the physiological range of membrane potentials. Each K_{2P} channel has its characteristic single channel behavior, allowing clear distinction among them. Studies show that K_{2P} channels are modulated by neurotransmitters and various physical and chemical factors indicating that these channels are important regulators of cell excitability.

B. Properties of K_{2P} Channel Subfamilies

TWIK family consists of TWIK-1, TWIK-2, and KCNK7 that are \sim 50% identical in amino acid sequences. TWIK-1 is abundantly expressed in the



FIGURE 1 Membrane topology and family tree of mammalian K_{2P} channels. Cylinders represent transmembrane segments and pore-forming (P) domains are indicated by loops that partially enter the membrane from the extracellular side. Six subfamilies can be identified based on the assumption that members of the same K_{2P} channel subfamily share greater than 40% amino acid identities. Two K_{2P} channel subunits assemble to form homo- or heterodimers. TRESK-1 and TRESK-2 may be orthologues.

brain whereas TWIK-2 is found more in peripheral tissues. TWIK-1 and TWIK-2 mRNAs are also expressed in DRG neurons (Medhurst *et al.*, 2001; Talley *et al.*, 2001). TWIKs may form functional homodimers but their single channel currents have not yet been recorded in any system (Lesage *et al.*, 1996b). KCNK7 does not form a functional channel as it is retained in the endoplasmic reticulum (Salinas *et al.*, 1999). The biological role of

TWIKs remains unknown at present. The possibility that TWIKs are intracellular channels has not yet been explored.

Tandem-pore Acid-Sensing K⁺ channel (TASK) family consists of TASK-1, TASK-3, and TASK-5. In situ hybridization and reverse transcriptase (RT)-PCR studies show that messenger RNAs (mRNAs) of TASK K_{2P} channels are expressed in many regions of the brain in rat and human (Medhurst et al., 2001; Talley et al., 2001). TASK-1 and TASK-3 are particularly highly expressed in cerebellar granule cells and motor neurons in the brainstem (Han et al., 2002; Berg et al., 2004). TASK-1 and TASK-3 form functional channels whose current-voltage relationship in high K⁺ medium is weakly inwardly rectifying. TASK-1 and TASK-3 are highly active at rest and therefore should contribute significantly to the background K^+ conductance. In neurons, such as the cerebellar granule neurons, TASK-1 and TASK-3 provide a large part of the background K⁺ conductance. TASK mRNAs are also expressed in sensory neurons, and their high acid sensitivity suggests that TASK-1 and TASK-3 may modulate excitability in these cells during inflammation. Our recent work shows that TASK-1 and TASK-3 can form a functional heteromer in cerebellar granule neurons (Kang et al., 2003). Motoneurons in brain stem also express heteromeric TASK channels containing TASK-1 and TASK-3 (Berg et al., 2004). Therefore, it seems likely that such heteromers are present in regions that coexpress TASK-1 and TASK-3. Such heteromerization would greatly increase the functional diversity of the K_{2P} channels, as TASK heteromers exhibit properties slightly different from those of TASK homomers (Kang et al., 2003). The possibility of heteromerization of other K_{2P} channel subunits needs to be examined. Extracellular acid, hypoxia, and agonists that act on receptors coupled to Gq/11 inhibit TASK-1 and TASK-3 (Buckler et al., 2000; Kim et al., 2000; Czirjak and Enyedi, 2002; Talley and Bayliss, 2002). TASK-1 and TASK-3 are activated by halothane (Patel et al., 1999). K⁺ efflux via TASK-1 and TASK-3 produces apoptosis in cerebellar granule neurons, as their inhibition promotes cell survival (Lauritzen et al., 2003). TASK-3 is overexpressed in cancer tissues, and the dominant negative mutant (TASK-3[G95E]) inhibited the oncogenic properties of TASK-3 (Mu et al., 2003; Pei et al., 2003). These studies show that TASK-1 and TASK-3 are involved in many important biological processes. TASK-5 does not form functional channels and its physiological role remains unknown (Kim and Gnatenco, 2001). It is interesting that TASK-5 mRNA is expressed highly in human adrenal gland and pancreas. The possibility that TASK-5 forms an intracellular channel needs further study.

TWIK-related K⁺ channel (TREK) family consists of TREK-1, TREK-2, and TRAAK. TREK-1 and TREK-2 mRNAs are expressed in many regions of the central nervous system, including DRG neurons, and in many

peripheral tissues. For example, TREK-1 mRNA is found abundantly in gut smooth muscle cells and TREK-2 mRNA is found in pancreas. TRAAK mRNA is expressed primarily in neurons of the central nervous system. TREK-2 is also expressed in brain astrocytes in culture and may participate in K⁺ spatial buffering (Gnatenco et al., 2002). In symmetrical 150 mM KCl, TREK-1 current shows a slightly outwardly rectifying current-voltage relationship, whereas TREK-2 and TRAAK are inwardly rectifying. At a physiological temperature of 37°C, TREK/TRAAK are very active and therefore will serve to stabilize the resting membrane potential and oppose depolarizing influences. TREK-1 knockout mice showed increased sensitivity to neuronal ischemia and epilepsy, suggesting that active background K⁺ conductance in certain brain regions is critical for neuroprotection (Heurteaux et al., 2004). As describe below in more detail, TREK/TRAAK are highly sensitive to temperature, acid, membrane tension, and free fatty acids such as arachidonic acid. TREKs are also inhibited by neurotransmitters that act on receptors coupled to Gs and Gq/11. These unique properties of TREKs strongly suggest that they may contribute to transduction of sensory information.

TWIK-related alkaline pH-activated K⁺ channel (TALK) family consists of TALK-1, TALK-2, and TASK-2 (Reves et al., 1998; Decher et al., 2001; Girard et al., 2001). TALK mRNAs are highly expressed in pancreas, and TASK-2 mRNA is expressed in a number of peripheral tissues including pancreas, kidney, and liver. All three members form functional channels with distinct single channel kinetics that should help distinguish them apart. The current-voltage relationships of all three channels are weakly inwardly rectifying in high K⁺ solution. TALK-1 and TASK-2 are highly active at rest at pH 7.3 whereas TALK-2 is generally inactive at the same pH (Kang and Kim, 2004). TALKs are highly sensitive to extracellular pH (pH_0) in the alkaline range as compared with TASKs that show higher sensitivity in the acidic pH range. TASK-2 is highly expressed inkidney and has been suggested to mediate K^+ efflux and volume regulation (Reyes *et al.*, 1998; Niemever et al., 2001; Barriere et al., 2003). Other than their role as background K⁺ channels, the physiological function of TALKs and their functional correlates in the native tissue have not yet been determined.

Tandem-pore halothane-inhibited K⁺ channel (THIK) family consists of THIK-1 and THIK-2 (Rajan *et al.*, 2001). THIK mRNAs are expressed in peripheral tissues and in certain defined regions of the brain including olfactory bulb and hypothalamus. THIK-1 forms a functional current in oocytes and COS-7 cells but THIK-2 does not. The single channels of THIK-2 have not yet been recorded, presumably due to its very small conductance. Therefore, THIK-1-like current has not yet been identified in any native tissues, and its physiological function remains to be determined. TWIK-related spinal cord K^+ channel (TRESK) family consists of TRESK-1 and TRESK-2 that may be orthologues (Sano *et al.*, 2003; Kang *et al.*, 2004). TRESK mRNA is expressed in many tissues including spinal cord and DRG neurons. Both TRESK-1 and TRESK-2 form functional channels that are active at rest, indicating that TRESKs also provide part of the background K^+ conductance. The single channel conductance of TRESKs is small and the kinetics is such that TREKs can be easily distinguished from other types of K^+ channels. The current–voltage relationship is linear for both TRESKs in 150 mM KCl. One unique aspect of TRESKs is that their carboxy termini are very short but their cytoplasmic linkers between the second and third transmembrane segments are very long. All other K_{2P} channels have long carboxy terminus but a short cytoplasmic linker. The biological role of TRESKs needs to be explored further.

C. Single Channel Properties of K_{2P} Channels

Expression of an ion channel mRNA in a tissue does not always indicate that a functional protein will be expressed. Therefore, it is crucial to determine the presence of a K_{2P} channel using antibodies or electrophysiological methods. Because of the numerous types of K^+ channels present in each cell type and lack of specific pharmacological inhibitors for K_{2P} channels, it is rather difficult to record a K_{2P} current and study it in isolation at the wholecell level. Furthermore, if several K_{2P} channels are expressed in a cell, it is nearly impossible to know which K_{2P} channels give rise to the measured whole-cell current. One practical way to assess functional expression of a K_{2P} channel is to record its single-channel openings in cell-attached or inside-out patches, although the regulation by intracellular factors may be lost in inside-out patches.

Most of the functional K_{2P} channels have now been characterized at the single-channel level in expression systems. Each K_{2P} channel exhibits distinct single-channel kinetics and conductance that should help to identify it in the native cell. Figure 2 shows an example of single-channel currents for K_{2P} channels expressed in COS-7 cells. Although no specific inhibitors of these K_{2P} channels are available, certain properties can be used to further confirm the identity of the K_{2P} channel. For example, TASKs are sensitive to pH_o and volatile anesthetics, and TREKs are sensitive to free fatty acid, pressure, and protons. Such methods have helped to identify the functional presence of TASK and TREKs in various neuronal cell types (Han *et al.*, 2002, 2003). Such methods have also helped to identify



FIGURE 2 Single channel openings of K_{2P} channels in COS-7 cells. Single channel currents from membrane patches of COS-7 cells transfected with various K_{2P} channel DNA are shown. Both single channel conductance and kinetics are sufficiently different from each other, and this should help to identify their functional expression in the native system.

the expression of TREK-1 and TREK-2 in DRG neurons, and will be necessary to identify other K_{2P} channels in various sensory neurons, and to study their roles in sensory transduction.

IV. EXPRESSION OF K_{2P} CHANNELS IN SENSORY NEURONS

Messenger RNAs of at least nine K_{2P} channels (TWIK1-2, TASK1-3, TREK1-2, TRAAK, KCNK7) are expressed in human DRG neurons (Medhurst et al., 2001). Three cranial sensory neurons (trigeminal, geniculate, and petrosal) that carry the somatosensory information from the oral cavity express TREK-1 mRNA (Matsumoto et al., 2001). Positive immunostaining of TREK-1 in mouse DRG neurons has also been reported (Maingret et al., 2000). Cholinergic motor nuclei of cranial nerves including the trigeminal nerve express high levels of TASK-1 and TASK-3 mRNA in the rat (Gu et al., 2002). Semiquantitative RT-PCR methods indicate that the expression of TWIK-2, TASK-1, TASK-2, TREK-1, and TRAAK mRNAs are much greater in DRG neurons than in the whole brain (Medhurst et al., 2001). TASK-2 immunoreactivity was observed in the spinal cord and small-diameter neurons of the dorsal root ganglia (Gabriel et al., 2002). TRESK mRNA is expressed in DRG neurons and spinal cord but not detected in the brain. Expression of other K_{2P} channels, such as TALK and THIK in DRG and other sensory neurons, has yet to be studied. These RT-PCR and in situ hybridization studies, however, do not directly show that all nine K_{2P} channel proteins are functional in DRG and other sensory neurons. It will be important to show plasma membrane expression of channel proteins using specific antibodies and by recording of K_{2P} currents.

V. FUNCTIONAL PROPERTIES OF K_{2P} CHANNELS

A. K_{2P} Channels are Active Background K^+ Channels

The classical inward rectifier (IRK; $K_{ir}2.x$) K⁺ channels have previously been thought to be the major background (resting or leak) K⁺ channels that set the resting membrane potential in excitable cells in the heart and brain. It is now clear that IRK is not the primary determinant of resting membrane potential, as K_{2P} channels are also expressed in many excitable cells. K_{2P} channels, such as TASKs and TREKs, which are expressed in neurons in certain regions are highly active near the resting membrane potential at physiological temperatures, and therefore may be the primary background K⁺ channels that oppose depolarization and stabilize the resting membrane potential in such cells (Kim *et al.*, 2000; Maingret *et al.*, 2000). The degree of contribution of K_{2P} channels to the background K⁺ conductance and resting membrane potential probably depends on the type and location of the neuron. In DRG neurons, inward rectifying K^+ channels, such as $K_{ir}2.3$, are expressed along with TREKs and TASKs (Baumann *et al.*, 2004). Therefore, it is likely that both K_{ir} and K_{2P} channels contribute to the resting K^+ conductance and help to maintain the negative resting membrane potential at approximately -55 mV. Certain K_v channels, such as members of the K_v1 and K_v2 families, in addition to their effects on repolarization and firing frequency, may also provide a fraction of the background K^+ conductance if they remain open after repolarization. Although the relative contribution of each K_{2P} channel to the resting K^+ conductance in many excitable cells including sensory neurons.

B. K_{2P} Channels Exhibit Diverse Functional Properties

1. Heat

One of the properties of TREKs that might be important in sensory transduction is their sensitivity to temperature (Maingret et al., 2000). In oocytes expressing TREK-1, the temperature range that modulates TREK-1 current was between $\sim 16^{\circ}$ C and 42°C, with a Q₁₀ of ~ 7 (change in activity upon 10°C change). In the oocyte system where ion channels are usually overexpressed, a low basal current can be observed at room temperature (22-24°C) and is reduced further at 16°C (Maingret et al., 2000). However, in isolated or cultured neurons, TREKs are normally not very active at room temperature but become progressively active as the temperature of the perfusion solution rises. TREK-1 activity increases with heat until about 42°C and then declines slightly above 42°C. Therefore, TREKs are likely to sense a temperature range between \sim 22°C and \sim 42°C. Figure 3A shows the effect of increasing the temperature of the perfusion solution on the whole-cell TREK-2 current in COS-7 cells. The temperature effects arerapid and quickly reversible. Similar effect of changing perfusion temperature can be observed in cell-attached patches as well (Fig. 3B). TREK-1 and TRAAK expressed in COS-7 cells also undergo such temperaturedependent changes in current with slightly different threshold for activation (Fig. 4). Available data indicate that TREKs begin to activate at ~24°C whereas TRAAK begins its activation ~30°C. Studies so far indicate that TREK/TRAAK are the only K_{2P} channels that are sensitive to heat/cold, as other K_{2P} channels, such as TASK, TALK, and TRESK, do not show temperature sensitivity.

Are native TREK/TRAAK expressed in sensory neurons also thermosensitive? Studies of TREK/TRAAK in sensory neurons have generally been



FIGURE 3 Temperature sensitivity of TREK-2. (A) Whole-cell currents were recorded from COS-7 cells transfected with DNA encoding TREK-2. Cell membrane potential was held at 0 mV to record outward current. After 1–2 min of equilibration, the temperature of the perfusion solution was increased from 24° C to 33° C and then back to 25° C. (B) Cell-attached patches were formed on COS-7 cells expressing TREK-2. Cell membrane potential was held at -30 mV. The temperature of the perfusion solution was increased gradually (left panel) or changed rapidly (right panel) as indicated by curves. Pipette and bath solutions contained 150 mM KCl. Top tracings show the change in temperature, as recorded directly from the bath solution using a bead thermistor. Reprinted from Kang *et al.* (2005) with permission from Blackwell Publishing.

limited to measurement of their expression at the mRNA level without functional studies. In a study, TREK/TRAAK channels were recorded in cell-attached patches of DRG neurons cultured from newborn rats (Fig. 5). The identity of each type of K^+ channel was confirmed by single channel conductance, and sensitivity to free fatty acids, acid/alkali, and membrane stretch, which are hallmark properties of TREK/TRAAK (Fig. 5). TREK/TRAAK were found in neurons with small, medium, and large-size cell bodies. At room temperature, the open probability was very low for all three channels. However, when the bath temperature was set at 37°C, the open probabilities of TREK/TRAAK increased



FIGURE 4 Temperature sensitivity of TREK-1 and TRAAK. Cell-attached patches were formed on COS-7 cells expressing TREK-1 or TRAAK. Cell membrane potential was held at -30 mV. The temperature of the perfusion solution was increased gradually from 24°C to 37°C. Pipette and bath solutions contained 150 mM KCl. Reprinted from Kang *et al.* (2005) with permission from Blackwell Publishing.

markedly, in keeping with the findings with cloned channels expressed in COS-7 cells. Among the three channels, TREK-2 was most abundant and most active. A TREK-2-like K⁺ channel was also recorded in chick DRG neurons (Fioretti *et al.*, 2004). The chick K⁺ channel was activated by arachidonic acid and acidic solution (pH 5.5), similar to the response observed with cloned TREK-2. Histamine caused activation of TREK-2like K⁺ channel via a cPLA2-sensitive pathway. Because TREKs are activated by unsaturated free fatty acids, such as arachidonic acid, histamine could be activating TREK-2 via a receptor-mediated generation of arachidonic acid (Fioretti *et al.*, 2004). These studies show that members of the TREK/TRAAK family are functionally expressed in DRG neurons.

The temperature sensitivity of TREK/TRAAK is close to those reported for TRPV3 and TRPV4 that are thermosensitive near the physiological range (Smith *et al.*, 2002; Xu *et al.*, 2002; Peier *et al.*, 2002b; Chung *et al.*, 2004). This compares with TRPV1 (VR1) that senses heat above ~43°C, TRPV2 (VRL-1) that senses temperatures above 50°C, and TRPM8 that



Dorsal root ganglion neuron

FIGURE 5 Functional expression of TREK/TRAAK in cultured DRG neurons. (A) Cellattached patches were formed on DRG neurons maintained at either 24°C or 37°C. Single channel openings of TREK-1, TREK-2, and TRAAK at 37°C are shown. (B) A graph shows the channel activities at 24°C or 37°C for the three channels. Each bar represents the mean \pm SD of five determinations and asterisks indicate a significant difference (p < 0.05). (C) The percent of patches containing TREK-1, TREK-2, and TRAAK in cell-attached patches of DRG neurons are plotted. (D) Inside-out patches containing TREK-2 were identified and maintained at 37°C. Single channel openings before and after application of arachidonic acid, negative pressure, or low-pH solution are shown. (E) A summary graph shows the degree of activation of TREK-2 at 37°C by arachidonic acid, negative pressure, or low-pH solution in the inside-out patches. Each bar represents the mean \pm SD of five determinations and asterisks indicate a significant difference (p < 0.05). (F) A photomicrograph shows cultured DRG neurons from 1-day-old rat. Reprinted from Kang *et al.* (2005) with permission from Blackwell Publishing.

senses temperature below $\sim 22^{\circ}$ C (Caterina *et al.*, 1997, 1999; McKemy *et al.*, 2002; Peier *et al.*, 2002a; Clapham, 2003). These TRP channels show steep temperature sensitivity, with a Q₁₀ of 10–20. Figure 6 illustrates the ranges of temperature sensitivity of various TRP ion channels and TREK. A study indicates that the temperature sensitivity of TRP ion channels is due to



FIGURE 6 Comparison of temperature sensitivity of TREK and TRP ion channels. The graph shows the range of temperature at which TRP ion channels and TREK-1 are active, as based on the results of published studies. Open circles indicate the thresholds of activation. TREKs are active at both warm and hot temperatures.

the shift in the voltage-dependent activation (Voets *et al.*, 2004). For example, the heat-activated TRPV1 is already highly active at room temperature at +100 mV, whereas it only starts to become active at \sim 35°C at -100 mV. Similarly, temperature-dependent shift in the activation curve is present for TRPM8 that is activated by cold. TREKs do not exhibit such voltage-dependence.

Repeated application of heat to TRPV1–3 sensitizes them to heat and lowers the threshold for activation. For example, the normal activation threshold of 50°C for TRPV2 (VRL-1) is lowered to 40°C by repeated heat application (Caterina *et al.*, 1999). Similar sensitizing effects may also occur for other thermosensitive TRP channels. Heat sensitivity of TRPV1 and perhaps other heat-sensitive channels are also modulated by other chemical and physical factors such as pH, inflammatory mediators (PGE2), peptides, cytokines, and G-protein–coupled receptor agonists. Therefore, the threshold temperature for activation of TRP channels may vary significantly depending on the state of the tissue. Although not tested, heat-sensitivity of TREKs could also be modified by various factors. Heat-sensitivity of TREKs is no longer observed in membrane patches, indicating that certain cytosolic components mediate the change in channel activity (Maingret *et al.*, 2000). The identity of the heat-sensitive factor remains to be determined.

How would TREKs affect heat transduction in sensory neurons? In DRG neurons, an increase in temperature from 24°C to 37°C would activate not only TRPV3 and TRPV4 but also TREKs. The depolarizing effect of the cation-selective TRPV3 and TRPV4 would be opposed by the activation of TREKs that would tend to stabilize the resting membrane potential. The threshold temperature for eliciting action potentials via activation of TRPV3 and TRPV4 may be lower in the absence of TREKs than in its presence, and

the sensitivity of the neurons to heat may be steeper as well. At temperatures above 43°C, several TRP channels including TRPV1 and TRPV2 and TREKs would also be active. In this scenario, TREKs would also be opposing the effect of the TRP channels and may reduce the action potential firing rate, and thus the sensation of heat. A change in TREK activity produced by various factors would clearly affect the degree of heat sensation transduced by sensory neurons. Perhaps, TREKs play a role in the adaptation of heat sensing.

Cooling decreases TREK current and this would provide a depolarizing influence on sensory neurons. TRPM8 (CMR1), originally cloned from rat trigeminal sensory neurons, is sensitive to temperatures in the range of 8-30°C (McKemy et al., 2002; Peier et al., 2002a). Cooling agents such as menthol and icilin also activate TRPM8. Therefore, nociceptive sensory neurons expressing both TREKs and TRPM8 may work in concert to transduce the "cool" and "cold" signals. In a subpopulation of primary sensory neurons excited by cooling and menthol, excitation occurred in part via closure of a background K^+ conductance (Reid and Flonta, 2001; Viana et al., 2002). Both central and peripheral temperature sensors regulate body temperature. The major central thermoreceptors are located in the posterior and preoptic regions of the hypothalamic regions. Several members of the K_{2P} channel family including TASKs and TREKs are also expressed in these regions (Talley et al., 2001). TREK-1 in particular is highly expressed in these regions of the hypothalamus and may help transduce cold sensing. Therefore, transduction of the thermal signal into cell excitability may involve both TRP and TREK ion channels.

2. Mechanosensitivity

The touch sensation begins at the level of the skin where sensory nerve endings transmit the signal from the skin to the central nervous system via spinal cord. Whether K_{2P} channels are expressed at the sensory nerve endings and help modulate the sensory transmission and adaptation is not yet known. One group of K_{2P} channels that might be expressed at the nerve terminals of DRG neurons is TREK/TRAAK, as they are highly expressed at the cell body. TREKs are highly mechanosensitive K^+ channels and may be involved in the modulation of touch sensation. Application of negative pressure to the membrane activates TREKs and TRAAK but not other K_{2P} channels. Cell swelling also activates TREKs, but the molecular detail of how this occurs is not clear. In DRG neurons, K^+ channels with properties similar to those of TREKs and TRAAK are also activated by mechanical stretch produced by application of negative pressure to the membrane (Su *et al.*, 2000). For TRPV4, a heat-sensitive channel that is also activated by cell swelling, the signaling pathway has been reported to involve phospholipase

A₂-mediated production of arachidonic acid and subsequent generation of 5',6'-epoxyeicosatrienoic acid via cytochrome P450 (Watanabe *et al.*, 2003; Vriens *et al.*, 2004). TREKs are activated directly by arachidonic acid and other unsaturated free fatty acids, most likely by acting on the carboxy terminus (Patel *et al.*, 1998; Kim *et al.*, 2001). Although not tested, metabolites of arachidonic acid could also activate TREKs. TREKs expressed in mechanosensitive sensory neurons would tend to shorten the depolarizing influence of Na⁺ channels and mechanosensitive TRP channels. In *Drosophila* bristle neurons, the TRP-related channel subunit NOMPC is required for mechanosensory transduction (Walker *et al.*, 2000). Whether TREKs are expressed in such cells is not known.

3. Acid/alkali-Sensing

Acid-induced pain occurs as a result of increased excitability of sensory neurons exposed to low-pH medium. Potential mediators are ASICs and TRP ion channels that are activated by acid and cause depolarization of the neuron. K_{2P} channels of the TASK and TALK subfamilies are highly sensitive to change in pH_o (Girard *et al.*, 2001; Kang and Kim, 2004). All members of the TASK subfamily are expressed in DRG and trigeminal neurons at the mRNA level, although actual channel currents have not yet been recorded (Medhurst et al., 2001; Baumann et al., 2004). Whether TALKs are expressed in these neurons is not yet known. Unlike TRPV1 that is rather insensitive to acid changes in the pH_0 6.0–7.3 range (Caterina et al., 1997), TASKs and TALKs exhibit sensitivity in the pH₀ 6–10 range, with some differences among the three K_{2P} channels (Kang *et al.*, 2003). TALK-1 and TALK-2 are highly sensitive in the pH₀ 7–10 range (Kang and Kim, 2004). The pH sensor in TASK-1 and TASK-3 is a histidine residue located at the pore domain (Kim et al., 2000; Rajan et al., 2000). The pH sensor in TALKs has not yet been identified. The pH sensitivities of TASKs and TALKs are shown in Fig. 7. Subtle differences in pH sensitivity among these K_{2P} channels could provide a sensitive detection of change in pH of the medium perfusing the sensory neurons.

A reduction in pH_o from 7.3 to 6.0 would produce a strong inhibition of all TASKs and increase neuronal excitability. Such a change in pH_o could depolarize the neurons to the point of threshold for firing, as it does in cerebellar granule neurons (Plant *et al.*, 2002). In DRG neurons, acid would close all TASK channels and facilitate depolarization by other stimuli, such as capsaicin, protons (pH < 6.0), or heat, which opens TRPV1. ASICs that are expressed in sensory neurons including DRG neurons would also be activated by acid and facilitate nociception as a result of increased excitability produced by Na⁺ influx. Therefore, acid-induced activation of TRPV1 and ASIC and subsequent depolarization would be further assisted



FIGURE 7 pH sensitivity of TASK and TALK K_{2P} channels. COS-7 expressing TASK-1, TASK-2, TASK-1/3 heteromer, TASK-2, TALK-1, and TALK-2 were used to test the effect of extracellular acid at the whole-cell level. The graph shows the high sensitivity of TASKs to acid and that of TALKs to alkali.

by inhibition of background TASK K^+ channels. At present, the degree of contribution of TASKs to the background K^+ conductance in any sensory neurons is not known, and therefore it is difficult to assess the relative role of TASKs in nociception mediated by tissue acidosis. In human, the relatively strong expression of TASK-1 and TASK-3 mRNA in DRG neurons suggest that TASK may be a significant player in nociception during inflammation.

The sensitivity of TREKs to intracellular acid is well known. Whether extracellular acid opens TREKs has not been tested rigorously. Given the high sensitivity of TREKs to intracellular acid, it seems likely that TREKs would be activated if pH were decreased sufficiently to levels that would also acidify the intracellular medium. Activation of TREKs by a very acid medium (pH 5.0) produces a strong activation of TREKs in COS-7 cells. TREKs in DRG neurons are also activated by highly acidic medium (pH 4–6) but not by mild acid. In TREKs, a glutamate residue in the carboxy terminus near the last transmembrane segment has been identified as the acid sensor (Honore *et al.*, 2002). TRAAK is activated by intracellular alkali and is unlikely to be involved in acid-induced pain sensation. The role of TRAAK in DRG neurons and in other cell types is not clear at present.

4. Volatile Anesthetics

Volatile anesthestics such as halothane and isoflurane act on many ion transport proteins including ion channels. In general, volatile anesthetics suppress neuronal excitability by inhibiting ion channels that cause depolarization (Na⁺ and Ca⁺ channels) and by activating ion channels that cause hyperpolarization (K⁺ and Cl⁻ channels). Studies show that volatile anesthetics are able to activate TREKs and TASKs in expression systems

(Patel *et al.*, 1999). These K_{2P} channels may thus be involved in halothanemediated hyperpolarization in the respiratory neurons of the brainstem and contribute to the anesthetic action of volatile anesthetics (Sirois *et al.*, 2000). In DRG neurons, volatile anesthetics, at clinically relevant concentrations, reduce the low voltage-activated Ca²⁺ current (Takenoshita and Steinbach, 1991). Whether halothane activates TREKs and TASKs expressed in DRG and other nociceptive sensory neurons has not been tested but seems likely. Anesthetics, such as halothane, can be used to alleviate pain without producing unconsciousness. One of the possible mechanisms of pain reduction could involve activation of TREKs and TASKs in sensory neurons and subsequent reduction of excitability. Whether volatile anesthetics modulate TRP channels has not been explored.

5. Lipids

Unsaturated free fatty acids, such as arachidonic, linoleic, linolenic, and oleic acids, strongly activate TREKs (Patel et al., 2001; Kim, 2003). Lysophosphatidic acids also strongly activate TREKs (Patel et al., 2001). Activation of TREKs by these lipid molecules would stabilize the membrane potential and make it more difficult for noxious and nonnoxious stimuli to depolarize sensory nociceptive neurons. However, there is no evidence that free fatty acids or lysophosphatidic acids mediate any sensory information. Free fatty acids themselves are generally ineffective in activating TRPV1. Receptor agonists, such as bradykinin, may produce arachidonic acid via phospholipase A₂ and activate TREK in DRG neurons and suppress excitability. However, bradykinin is well known for its pain producing action by activating and sensitizing sensory neurons. This effect of bradykinin may be due to activation of TRP ion channels via 12-HPETE, a lipoxygenase metabolite of arachidonic acid (Shin *et al.*, 2002), and by inhibition of TREK/ TASK via PLC and Gq/11 (Fig. 8). Bradykinin-induced activation of TRPV1 is also enhanced in the presence of PKCE, suggesting that phosphorylation of VR1 is critical (Premkumar and Ahem, 2000). Whether bradykinin modulates TREKs in DRG neurons is not yet known. Since bradykinin depolarizes the DRG neuron, the inhibitory effect of bradykinin on TREKs via Gg/11 may be stronger than the activation via generation of arachidonic acid. These signaling pathways need to be tested directly in DRG neurons.

Certain lipid molecules can activate TRP ion channels other than TRPV1. For example, the light-sensitive TRP and TRPL in *Drosophila* are activated by polyunsaturated free fatty acids, and TRPC3 and TRPC6 are directly activated by diacylglycerol (Chyb *et al.*, 1999; Hofmann *et al.*, 1999). Therefore, receptor agonists that generate free fatty acids and diacylglycerol may transduce various sensory signals by activating specific TRP ion channels. On other TRP channels, the effect of diacylglycerol is via activation of



FIGURE 8 Potential signaling pathways for modulation of TREK and TRP ion channels in DRG neurons by receptor agonists. Agonists act on Gq/11-coupled receptor and modulate TREK and TRP ion channels via phospholipase C-mediated production of second messengers that include diacylglycerol, protein kinase C, and Ca^{2+} . Depletion of membrane PIP2 by PLC may also serve as a signal to modulate channel function. Agonists, such as bradykinin, may also act on G-protein–coupled receptors to activate PLA2 to generate arachidonic acid that may directly or indirectly modulate TREK and TRP ion channels.

protein kinase C that either inhibits (TRPC4 TRPC5) or activates (TRPV1) TRP ion channels. Because diacylglycerol may inhibit TREK activity (Chemin *et al.*, 2003), the net effect of diacylglycerol on sensory neurons would be the sum of the effects on both TRP and TREK channels.

Many sensory neurons express both cannabinoid CB1 receptors and TRPV1. Anandamide is a lipid agonist of CB receptors and has antinociceptive properties. Anandamide is also a partial agonist of TRPV1 and a mild activator of TREK (De Petrocellis *et al.*, 2001; Olah *et al.*, 2001; Gnatenco *et al.*, 2002). Therefore, the antinociceptive actions of anandamide may be due in part to activation of TREK. Capsaicin acting on TRPV1 and subsequent elevation of intracellular $[Ca^{2+}]$ has been reported to cause synthesis of anandamide in DRG neurons. Anandamide thus generated would act on CB receptors to suppress excitability and reduce nociceptive transmission as part of a natural response to pain.

6. G-Protein–Coupled Receptors

Receptor agonists that act on μ - and δ -opioid receptors, α_2 receptor, 5HT_{1a} receptor, and GABA_B receptor are believed to produce antinociceptive effects via activation of voltage-gated, ATP-sensitive, Ca²⁺-activated and/or G-protein–gated K⁺ channels via Gi/o (Ocana *et al.*, 2004). TASKs

and TREKs are targets of G-protein-coupled receptors. Receptor agonists that act on Gq/11, however, inhibit background K⁺ channels such as TREK and TASK. The exact mechanism of inhibition is not clear but is likely to be via molecules generated by PLC such as inositol trisphosphate, diacylgly-cerol, and/or by reduction of membrane [PIP2] (Chemin *et al.*, 2003). TREKs are also inhibited by agonists that act on Gs-coupled receptors via phosphorylation by protein kinase A on a serine residue in the carboxy terminus (Patel *et al.*, 1998). Thus, an important mechanism by which receptor agonists increase cell excitability is via inhibition of background K⁺ channels such as TASK and TREK (Fig. 8).

DRG neurons express various Gq/11-coupled receptors including glutamate, opiate, bombesin, bradykinin, substance P, and endothelin receptors. Some of these receptor agonists are involved in transmission of pain and stress at the level of the spinal cord, presumably in part via activation of nonselective cation channels. Agonists that act on these receptors may also increase excitability by inhibiting TASK and TREK currents and thereby further facilitate sensory transmission. Bradykinin and nerve growth factor are pro-algesic molecules that act on receptors coupled to Gq/11 and stimulation of PLC. They appear to sensitize sensory nerve endings to noxious stimuli and enhance pain sensation by reduction of [PIP2] and "releasing" TRPV1 from PIP2-mediated inhibition (Chuang *et al.*, 2001; Prescott and Julius, 2003). Thus, the threshold for activation of TRPV1 by protons, capsaicin, or lipids is shifted to more negative membrane potentials by a reduction of [PIP2] in the membrane. Whether other agonists that also act on Gq/11-coupled receptors sensitize TRPV1 has not been tested.

The sensitization of neurons to agonists (or disinhibition of TRPV1) provided by PLC-mediated degradation of PIP2 is not a common observation among ion channels. More common observation is that the interaction of ion channel with membrane PIP2 stabilizes the open state of the channel and increase the open probability. TRPM7 is a good example of how PIP2 upregulates and its degradation downregulates channel activity (Runnels et al., 2002). Similar phenomena on the effect of PIP2 are observed for inwardly rectifying (IRK), G-protein-gated (GIRK), neuronal M, and ATP-sensitive K⁺ channels (ROMK). PIP2 and other phospholipids may also be important in regulating the gating of TREK-1 and perhaps TREK-2 as well, as both possess the phospholipid-sensing domain that interacts with the plasma membrane lipids (Chemin et al., 2004). The phospholipids-TREK interaction also seems critical for channel modulation by membrane tension and acid. Whether phospholipids also affect the heat sensitivity of TREKs has not yet been tested. Thus, some of the agonists may inhibit the background K⁺ current provided by TASK and TREK and help to sensitize the nerve terminals to noxious stimuli by an effect on membrane PIP2 concentration.

VI. SUMMARY

Sensory neurons express a set of ionic channels that are able to detect various sensory stimuli, and transmit the sensory signal in the form of trains of action potentials. One or more of these ion channels belong to the K⁺ channel family known as K_{2P} channels. A number of K_{2P} channels are now known to be expressed in DRG and in other sensory neurons. K_{2P} channels not only provide part of the background K⁺ conductance important for setting and stabilizing the resting membrane potential, but they also regulate cell excitability in response to various biologically relevant chemical and physical factors. TRP ion channels are considered to be cellular sensors for temperature, touch, pain, osmolality, pheromones, and taste, as they are directly activated by these stimuli, and depolarize sensory neurons. Because K_{2P} channels, such as TASK and TREK, are also sensitive to similar stimuli, including heat and cold, membrane tension, pH, and osmolality, both K_{2P} and TRP ion channels are likely to be involved in regulating the excitability of sensory neurons and thus in the sensory transduction.

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CHAPTER 13

Finding Sensory Neuron Mechanotransduction Components

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References

I. OVERVIEW

Almost all primary afferent sensory neurons including nociceptors are mechanosensitive, it is surprising that we still do not understand the molecular basis of the process whereby nociceptive and nonnociceptive sensory neurons transduce mechanical signals. This process termed sensory neuron mechanotransduction is the subject of this chapter. In particular, we will review what is known about this process in primary afferent nociceptors and examine the experimental approaches that scientists have used and are using to identify potential molecules involved in sensory neuron mechanotransduction. The applicability of knowledge gained about molecules involved in mechanotrasnduction in lower organisms will be discussed. It is clear that identification of tractable molecular targets that are required for transduction of mechanical stimuli in nociceptors would represent a significant step in the development of new analgesic drugs.

II. INTRODUCTION

Sensory neuron mechanotransduction is the process whereby primary sensory neurons transduce a mechanical stimulus into an electrical signal. Mechanical stimuli that cause pain might be an intense, such as pinching of the skin, traumatic injury or under neuropathic conditions brush or light pressure. In the former case it is believed that specialized sensory neuron nociceptors detect intense mechanical stimuli and their activation alone is responsible for the acute mechanical pain. In the latter case neuropathic pain after nerve injury is thought to lead to a reorganization of synaptic connections made by low-threshold mechanoreceptors in the spinal cord (Lewin et al., 2004). Under these circumstances brush activation of low-threshold mechanoreceptors can lead to pain because of altered synaptic connectivity in the spinal cord. We will briefly review what is known about vertebrate primary sensory neurons that transduce mechanical signals, however, a more extensive review of this literature is found in a topical review (Lewin and Moshourab, 2004). What is perhaps the most interesting open question in sensory physiology is what molecules do both nociceptive and nonnociceptive sensory neurons use to detect mechanical stimuli. This is not a trivial experimental challenge as we will attempt to make clear here. We will focus on the methodologies that have been used to try and identify molecular components of the mechanotransducer. The relative merits of these methodologies will be compared and the data implicating the various candidate molecules so far identified evaluated.

III. MECHANICAL NOCICEPTORS AND OTHER RELEVANT MECHANORECEPTORS

Most cutaneous sensory neurons respond to mechanical stimuli (Lewin and Moshourab, 2004), although there is also evidence for small population of so-called mechanically insensitive or silent nociceptors (Meyer *et al.*, 1991;

13. Finding Sensory Neuron Mechanotransduction Components

Kress et al., 1992; Schmidt et al., 1995; Michaelis et al., 1996). In general, low-threshold mechanoreceptors that normally do not subserve pain have encapsulated endings in the skin where mechanical stimuli are transduced (Fig. 1). Myelinated $A\beta$ -fibers with large axon diameters are generally lowthreshold mechanoreceptors and can be divided into two main groups. Rapidly adapting (RA) mechanoreceptors respond exclusively to movement of skin but not to static indentation. In man rapidly adapting sensory afferents have been classified as RA type I and RA type II. RA type I fibers innervate the Pacinian corpuscle (Munger and Ide, 1988) and RA type II are believed to innervate Meissner-like corpuscles in glabrous skin (Munger et al., 1979). In the hairy skin, most often studied in experimental animals, RA fibers with type II responses innervate hair follicles where they form palisade (sometimes called lanceolate endings) or Ruffini type endings. RA fibers of both types are tuned to detect movement, at high (RA type I) or low frequencies (RA type II) (Fig. 1). Slowly adapting (SA) mechanoreceptors sense movement and indentation of the skin, their endings are within Merkels disks (SA I) at the border of epidermis and dermis or they are associated with Ruffini corpuscles (SA II) in the deeper layers of the dermis, SA II fibers preferentially detect skin stretch (Iggo and Muir, 1969).

Primary sensory neurons with thin myelinated $A\delta$ -fibers innervating the skin can also be classified into two groups as well. The high-threshold mechanoreceptors or A-fiber mechanonociceptors (AM fibers) have intraepidermal free nerve endings that are often unmyelinated near the ending (Fig. 1). Some associate with Schwann cells or keratinocytes (Kruger *et al.*, 1981). AM neurons in general are activated by more intense mechanical stimuli (Koltzenburg *et al.*, 1997).

D-hair receptors are low-threshold mechanoreceptors. They are sensitive to very small forces and are associated in the cat with the movement of down hairs, hence the term D-hair receptor (Brown and Iggo, 1967; Koltzenburg *et al.*, 1997). The location and morphology of D-hair fiber endings in the skin is so far unknown. Circumstantial evidence suggests that the endings of D-hair mechanoreceptors are on hair follicles perhaps of the Ruffini type (Stucky *et al.*, 1998). D-hair receptors have been described in cats, primates, rodents, and man (Burgess and Perl, 1967; Perl, 1968; Adriaensen *et al.*, 1983; Lewin and McMahon, 1991; Lewin *et al.*, 1992; Leem *et al.*, 1993; Koltzenburg *et al.*, 1997).

Unmyelinated C-fibers form another group of mechanosensory neurons. They account for 60–70% of all primary afferent neurons and because of their lack of myelin have very slow conduction velocities (<1 m/s). Some C-fibers have very low mechanical thresholds so-called C-LT (C-fiber low-threshold mechanoreceptors) but most have high-mechanical thresholds and in addition may respond to noxious thermal stimuli (Bessou and Perl, 1969).



FIGURE 1 Typical response properties of mouse mechanoreceptors from the saphenous nerve to a standardized 2 s ramp and hold indentation stimulus of $150 \ \mu m$ are shown. In the center a schematic diagram of the dorsal root ganglia depicts the approximate cell size and myelination state of the different mechanoreceptors (thick cell wall indicates myelinated neurons). The mechanoreceptors can be divided into two major groups, low-threshold mechanoreceptors all robustly respond to the ramp phase of the indentation stimulus given to the skin. These neurons are either large myelinated sensory neurons, such as rapidly, or slowly adapting mechanoreceptors (RA or SA receptors), or medium sized sensory neurons with thin myelinated axons, the so-called D-hair mechanoreceptor. Mechanoreceptors with essentially no response to the stimulus. These neurons sometimes have thin myelinated axons and are called in this case A-fiber mechanonociceptors (AM) or have non-myelinated axons and are therefore called C-fiber mechanonociceptors (C-M). The nociceptive population can be further divided according to

The so-called free nerve endings of these C-fibers are found in the connective tissue of the dermis and epidermis, where they are sometimes associated with structures such as sweat glands (Munger and Ide, 1988).

Much is known therefore about the microscopic structures containing mechanotransducing ion channels and their associated proteins in the skin of mammals. However, little has been established about the mechanisms, the identity of the transducing proteins, which underlie the transduction of the mechanical stimulus into an electrical signal (Lewin and Stucky, 2000).

IV. PHYSIOLOGY OF TRANSDUCTION

Of all the mechanoreceptor types described above (Fig. 1) it is only the cat Pacinian corpuscle (RA type I) mechanoreceptor where direct recordings of the mechanically activated receptor potential have been made in the intact system. This is mainly because the site of transduction is far away from the cell body and the size of the peripheral endings are too small for direct intracellular recordings (Fig. 2). Much of the early work on the Pacinian corpuscle was carried out by Loewenstein and his colleagues during the 1960s and 1970s. Loewenstein took advantage of the large size of the cat Pacinian corpuscle receptor ending to make high-resolution recordings of the mechanically activated receptor potential in an isolated corpuscle. Single Pacinian corpuscles were placed in a small chamber and an extracellular potential was measured at the point where the afferent axon exits the corpuscle. Very small mechanical stimuli applied to the corpuscle evoked a nonregenerative depolarization that could be measured when action potential generation was blocked with a local anaesthetic. The very high speed of this transduction event and the fact that the magnitude of the depolarization of the receptor ending was graded suggests that the receptor potential is

their response to noxious thermal stimuli, those neurons responding to noxious heat in addition to mechanical stimuli are called C-fiber mechanoheat nociceptors (C-MH). The approximate incidence (percentage of total cutaneous sensory neurons) is indicated next to its name. Note all action potential records are real recordings made using the mouse skin–nerve preparation. The firing rates and patterns of discharge of the chosen examples are typical for the receptor type indicated. For example, AM neurons typically have higher rates of firing than do C-M or C-MH nociceptors. Anatomical structures associated with the various types of mechanoreceptor endings in the skin are also schematically illustrated in this figure. Thus, RA mechanoreceptors have been described as having endings in one of three types of structure within the skin, Pacinian corpuscle, or Meissners corpuscle, or specialized endings associated with hair follicles. SA mechanoreceptors have been described as being associated with Merkel cells at the dermal– epidermal boundary or have endings within Ruffini corpuscles. Nociceptors are in general thought to have free nerve endings within the dermis or epidermis.



FIGURE 2 A schematic representation of the architecture of primary sensory neurons is shown with the places where it is possible to record the transduction of mechanical signals illustrated. Note that intracellular electrophysiological recordings are only possible from the cell soma. It is known that the membrane of the cell can be sensitive to thermal stimulation at least in culture, but *in vivo* robust mechanically evoked responses are only seen after stimulation of the sensory ending within the skin. The distance between the site of transduction and the cell soma are several orders of magnitude larger than the size of the axon or cell body. This fact makes intracellular recording of transduction events practically nonfeasible. Note that the two ends of the same axon are specialized to quite different jobs, transduction in the skin and transmission of sensory information via fast synapses in the spinal cord.

supported by specialized mechanically gated channels (Loewenstein and Skalak, 1966). Similar receptor potentials have been measured from another encapsulated receptor, the muscle spindle stretch receptor (Hunt, 1990), but other mechanoreceptors, including all mechanical nociceptors, have proven too small or too inaccessible to allow recordings of the mechanically gated receptor potential to be made in situ. Studies on mechanoreceptors in invertebrates, for example, crayfish muscle stretch receptors, the spider slit mechanoreceptor, and most recently the *Caenorhabditis elegans* body touch receptor have revealed that receptor potentials similar to those observed in Pacinian corpuscles are also observed in these mechanoreceptors (Loewenstein and Skalak, 1966; French, 1992; Hamill and McBride, 1996; O'Hagan et al., 2005). The nature of the mechanically gated channels in mammals and their mechanism of gating has remained a mystery in the vertebrate system. It must also be pointed out that of all the high-resolution electophysiological recordings that have been made in various preparations in which the receptor potential has been directly measured, all have been made from nonnociceptive mechanoreceptors. Thus, so far no one has managed to make recordings of the receptor potential from identified nociceptive neurons in any species.

V. MOLECULAR IDENTITY OF THE TRANSDUCER

Strategies for identifying the molecular nature of the mammalian mechanotransducer are the main topic of this chapter. The most successful approach to identify molecules necessary for mechanotransduction has been to carry out genetic screens for mutant animals that display a sensory deficit. This is a so-called reverse genetic approach where one first identifies a mutant with a specific phenotype and then clones the responsible gene. This approach applied to mechanotransduction has most successfully been applied in the nematode worm *C. elegans* and fruitfly *Drosophila melanogaster*. Such approaches have not yet been applied to identify mechanotransduction genes in vertebrates. The results of the genetic screens directed to find mechanotransduction components in both *C. elegans* and *Drosophila* are summarized below.

A. Caenorhabditis elegans

The relatively simple worm C. elegans is an ideal animal model to study touch mechanotransduction. Every single cell in C. elegans has been counted and given a name and of the very few neurons present in this organism (302), six have been shown to be sensory neurons that specifically detect and transmit touch sensation from the body wall. These neurons have a single long process that runs underneath the hypodermis and the axon is ensheathed by a dense extracellular matrix called the mantle. Within the sensory axon process, electron miscroscopy reveals a bundle of 15-protofilament microtubules. They seem to be cross-linked among themselves and the distal ends terminate directly below the cell membrane (Chalfie and Thomson, 1979; Hodgkin, 1991; Chalfie and Jorgensen, 1998). The C. elegans mec genes (mec stands for mechanosensory abnormal), were discovered by screening mutagenized worms for individuals responding poorly or not at all to body touch. This behavioral screen led to the identification of many genes (18 in total) that when mutated caused a phenotype characterized by reduced or absent responses to body touch. Of the identified mutant lines, twelve represent mutations in single genes that appear to leave the morphology of the touch cell intact. For this reason these mec genes were seen as attractive candidates as participants in the transduction event itself



FIGURE 3 Schematic drawing of the putative composition and of the body touch receptor mechanosensory complex in *C. elegans.* The ion channel subunits MEC-4 and MEC-10 are thought to form the core of the pore-forming channel that is attached simultaneously to extracellular matrix components consisting of MEC-1, MEC-5, and MEC-9. In addition two other MEC proteins MEC-2 and MEC-6 are also thought to be essential for the channel to function properly and MEC-2 may also link the membrane associated complex to the intracellular cytoskeleton made up of two specialized microtubules MEC-7 and MEC-12. On the left the closed inactive complex is shown. On the right the open complex is shown, the mode of opening is thought to be via the transfer of force through the extracellular matrix that leads to a change in ion channel conformation to an open state that is highly selective for sodium ions.

(Fig. 3). In 1991, Driscoll and Chalfie presented evidence for the first time that one of these *mec* genes might code for an ion channel, this ion channel protein was designated MEC-4 (Driscoll and Chalfie, 1991). Certain point mutations in the *mec-4* gene lead to a specific amino-acid exchange in the protein that causes the cells expressing this mutated channel to die through necrosis. For this reason the MEC-4 protein was also described as a degenerin, as mutations in this channel protein can lead to neuronal degeneration, probably by overloading the cells with calcium to which the mutated channels are permeable (Bianchi et al., 2004). The mec-4 and mec-10 genes were both found to cause degeneration when mutated and were quickly identified as the probable ion channel core of the mechanotransducer. The MEC-4 and MEC-10 proteins both belong to the degenerin/epithelial amiloride sensitive Na⁺ channels (DEG/ENaC) superfamily of ion channels. They are permeable to Na⁺, inhibited by amiloride, and not voltage-gated (Waldmann and Lazdunski, 1998). MEC-4 and MEC-10 like all other degenerin members have two transmembrane domains, with both carboxy- and aminoterminal being cytoplasmic. The extracellular loop of these proteins is characterized by several cysteine-rich domains (Lai et al., 1996).

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The now widely accepted model that has been assembled from data arising from the genetic screen is that MEC-4 and MEC-10 build the pore-forming core of the mechanosensory complex. However, without the help of additional subunits proteins, also identified in the screen for touch insensitive mutant worms, this channel does not function properly (Goodman *et al.*, 2002; O'Hagan *et al.*, 2005). Thus heterologous expression of the *C. elegans* MEC proteins in *Xenopus* oocytes revealed that MEC-2 is a membrane protein that is perhaps necessary for MEC-4 activity. It is however notable that amiloride sensitive currents could only be measured in *Xenopus* oocytes when a mutated version of the MEC-4 channel that causes neurodegeneration (MEC-4d) is used (Goodman *et al.*, 2002; Bianchi *et al.*, 2004). MEC-2 exhibits close sequence homology with stomatin, a putative regulator of ion flux in human red blood cells (Huang and Chalfie, 1994). Because stomatin is associated with lipid rafts, MEC-2 might also be a raft protein (Salzer and Prohaska, 2001).

Another important component of the MEC-complex is MEC-6, a single membrane spanning, almost completely extracellular protein. MEC-6 shows similarities in amino acid sequence to the vertebrate paraoxonase/arylesterase, which are encoded in man and mice by the paraoxonase gene cluster (PON) and are named PON 1-3. All of the PONs prevent lipid oxidation (Primo-Parmo et al., 1996; Draganov et al., 2000; Ng et al., 2001). So far it has not been possible to attribute a detailed function to MEC-6, but it has been demonstrated that the opening of ion channel complexes consisting of MEC-4d/MEC-10 expressed in oocytes is greatly facilitated in the presence of MEC-6 and MEC-2 (Chelur et al., 2002). Thus, MEC-4, MEC-10, MEC-6, and MEC-2 function synergistically; and when all are present, amiloride-sensitive Na⁺-currents carried mutated channels are increased more than 200-fold compared to expression of MEC-4d and MEC-10d alone (Chelur et al., 2002). Proof that MEC-4, MEC-10, MEC-2, and MEC-6 are actually necessary for the transduction of mechanical stimuli in vivo was provided by direct recording of mechanically gated inward currents is sensory neurons in the worm in vivo (O'Hagan et al., 2005). Examples of inward currents evoked in C. elegans with indentation stimuli are shown in Fig. 4.

In addition to the central components of the mechanotransduction complex, mutations in several other genes also led to behavioral touch insensitivity. Thus, three additional *mec* genes (*mec-5*, *mec-1*, and *mec-9*) were found to code proteins associated with the extracellular matrix. The MEC-5 protein is a collagen but has little similarity to other collagens, the only structural homology that MEC-5 shares with other collagens are Gly-X-Y repeats. Mutations that disrupt the Gly-X-Y repeats lead to touch-insensibility. MEC-1 and MEC-9, both required for protein–protein interactions, contain epidermal growth factor (EGF) domains and so-called Kunitz protease inhibitor domains.



FIGURE 4 Mechanically gated receptor currents (MRCs) recorded from wild-type and mutant *C. elegans* body touch sensory neurons. The MRCs were recorded in the voltage clamped cell soma of PLM sensory neurons in the intact worm. In (A) representative MRCs from cells recorded in *mec-7(u142)*, *mec-10(u20)*, *mec-4(e1339)*, and *mec-4(u2)* mutants are shown. Stimulus amplitude (in nN/µm²) is noted above each trace, which is an average of 6–20 sweeps. In (B) the quantitative data from many recordings of the peak MRC amplitude as a function of PLM genotype is shown. In (C) the average current–voltage relation for the MRC in wild-type, *mec-4(e1339), mec-4(u2)*, and *mec-10(u20)* normalized to the current at –94 mV is shown. A null mutation of the microtubule gene *mec-7(allele u142)* leads to the PLM sensory MRC exhibiting a lower pressure sensitivity than wild-type. (D) Normalized peak MRC is plotted versus pressure for three *mec-7(u142)* recordings. Smooth line (gray) is a global fit to the data with a Boltzmann function (*P1/2* = 10.9nN/µm², *P*_{slope} = 3.3nN/µm²); the Boltzmann curve for wild-type (black) is shown for comparison. Reproduced with permission from O'Hagen *et al.* (2005).

However, the latter shows no enzyme activity (Du *et al.*, 1996). Absence of MEC-1, for example, results in a loss of attachment of processes near the cell surface but not a complete loss of touch sensibility (Chalfie and Sulston, 1981).

Intracellular components of the complex are α and β tubulin, encoded by *mec-12* and *mec-7*. Both required for the formation of the 15-protofilament

microtubules. Absence of one of these two *mec* genes leads to a loss of normal diameter microtubules and a loss of touch sensitivity (Savage *et al.*, 1994; Fukushige *et al.*, 1999). Recordings from single sensory neurons in worms carrying a *mec*-7 mutation do not lead to a complete loss of the mechanosensory potential (O'Hagan *et al.*, 2005). Therefore, one can observe a touch insensitive phenotype without abolishing completely the mechanosensitivity of the neurons that detect the stimulus.

Other mechanosensory neurons in the nematode include those required for nose touch responses. A genetic screen was carried out for worms that would avoid a hypo-osmotic stimulus. Two of the genes required for this behavior *Osm-9* (Osmotic avoidance abnormal protein 9) and *Ocr-2* (OSM-9 and capsaicin receptor-related) code for a TRP channel (see the following section), loss of function mutants also lost their ability to respond to nose touch (Colbert *et al.*, 1997; Tobin *et al.*, 2002; Tobin and Bargmann, 2004). It has therefore been hypothesized that this channel might be capable of detecting both mechanical displacement and at the same time be osmosensitive. The sensory neurons reponsible for detecting nose touch and osmotic stimuli are so-called ciliated mechanoreceptors, whereas the body touch receptors are not. This means that dendritic-like processes that contain intracellular organelles typical of cilia probably are the sites of stimulus detection.

B. Drosophila melanogaster

The key molecular players for mechanotransduction in *Drosophila* are less extensively investigated than in *C. elegans.* There have been two types of mechanosensory neuron described in *Drosophila*, the type I and type II neurons. The type I neurons are ciliated mechanosensory neurons that underlie mechanosensory responses to bristle movement as well as a primitive hearing sense located in the chordotonal organ. The type II neurons are multidendritic neurons found in *Drosophila* larvae, which spread their dendrites underneath the epidermis and have terminal endings embedded in the cuticle (Jarman, 2002). Type I neurons exemplified by the body bristle receptors have a ciliated dendritic process that penetrates the external bristle. The organ is composed, in addition, of three support cells that secrete an endolymph that has a high potassium- and a low calcium concentration (Grunert and Gnatzy, 1987). Movement of the bristle leads to dendrite displacement, which in turn induces a very fast opening of cation channels and neuronal depolarization (Corfas and Dudai, 1990).

Maurice Kernan and colleagues pioneered genetic screens for touch insensitive flies (Kernan *et al.*, 1994; Kernan and Zuker, 1995). To date several genes have been identified as being necessary for bristle touch and for chordotonal organ function. Three of these genes NompC, Nanchung, and Inactive code for putative ion channel proteins that are again members of the TRP family (Walker et al., 2000; Kim et al., 2003; Gong et al., 2004). The Nanchung and Inactive proteins are necessary for Drosophila hearing and have also been shown to be activated by hypo-osmotic stimuli when expressed in a heterologous system. It is however not clear if that relates directly to their in vivo gating by mechanical stimuli. In addition to these ion channels NompA has been shown to be an extracellular matrix protein that might be required for attaching the sensory cilia to the bristle to facilitate gating (Chung et al., 2001). NompA and C derive their names from genetic screens detecting no mechanoreceptor potential (nomp) mutants (Kernan et al., 1994). NompA is a transmembrane protein, containing the cysteine-rich zona pellucida (ZP) domain, probably for protein-protein interaction and several plasminogen N-terminal (PAN) modules (Chung et al., 2001). NompC presumably interacts with the cytoskeleton or other intracellular molecules via its 29-ankyrin repeat (Walker et al., 2000). On the outer side of the cell membrane, NompC exhibits only a small extracellular domain, suggesting that for a complete mechanosensory channel complex, other, so far unidentified proteins are waiting to be discovered.

A genetic screen was designed by Tracey and colleagues to find nociceptor behavior defective fly larvae. In this screen a new *TRP* gene was discovered that apparently is required for the avoidance of noxious temperature as well as noxious mechanical stimuli. This gene was named *painless* and is localized in the dendritic terminals of multidendritic neurons in the fly larvae (Tracey *et al.*, 2003). This is the only TRP involved in invertebrate mechanosensation that is not expressed in ciliated mechanosensory neurons.

In summary, genetic screens in invertebrates have been very good at identifying candidate molecules including channel proteins that are involved in mechanotransduction. In the next section we will discuss the success of using such information to identify genes in mammalian genomes that have a functional role in the detection of low- or high-threshold mechanical stimuli by sensory neurons. In addition we will discuss the application of other nongenetic techniques to identify such molecules.

VI. IDENTIFICATION OF MOLECULES REQUIRED FOR VERTEBRATE SENSORY MECHANOTRANSDUCTION

A major obstacle one faces while studying mechanotransduction in mammals is the relative abundance of sensory endings in which the transduction takes place is very low (Fig. 2). This makes the use of protein purification and other classical biochemical to purify transduction components very difficult. It is therefore not surprising that the application of molecular biology to the task is starting to yield the first insights into molecular mechanisms of mechanotransduction in mammals.

VII. CANDIDATE GENE APPROACHES

It is a common practice to start the search for new candidate genes by searching the genome for gene orthologs of known mechanotransduction components identified in other species. However, one has to consider that not all genes have conserved orthologs in distantly related species. For example, in *C. elegans* 10–25% of its genes are unique to the nematode, and other genes present in other invertebrates and vertebrates do not exist in the worm (Ruvkun and Hobert, 1998).

VIII. ARE THERE VERTEBRATE mec GENES INVOLVED IN SENSORY MECHANOTRANSDUCTION?

One common thread that comes from genetic screens for molecules involved in mechanosensation in invertebrates is that transduction may be carried out by a complex of proteins (Ernstrom and Chalfie, 2002). The results of these screens led to the hypothesis that ion channels detect mechanical strain on the membrane by virtue of their attachment to cytoplasmic and extracellular molecular elements (Lewin and Stucky, 2000; Ernstrom and Chalfie, 2002). One corollary of this hypothesis is that mechanotransduction ion channels when expressed alone may not necessarily be directly gated by membrane curvature. Instead, a multimolecular complex in which stress is applied via extracellular links to the channel to facilitate gating has been proposed. In this section we will concentrate on studies in mice where the function of putative mechanotransduction molecules homologous to the *mec* genes identified in the *C. elegans* body touch screen have been studied in identified mechanoreceptors.

It was obvious that if a member of the DEG/ENaC superfamily of sodium channels functions at the core of the metazoan mechanotransducer then it might be the case that a related channel functions in mammalian mechanotransduction. At the time that the MEC-4 and MEC-10 proteins were discovered in *C. elegans*, Canessa and colleagues cloned a highly related vertebrate channel from the mammalian kidney that codes for an amiloride sensitive sodium channel (Canessa *et al.*, 1993). This was the founding member of the epithelial sodium channel family (ENaC) and belongs to

the same family as MEC-4, now called the DEG/ENaC superfamily (Kellenberger and Schild, 2002). Amiloride sensitive epithelial sodium channels form a subgroup consisting of four members (α , β , δ , γ ENaCs) that can form heteromeric channels (Kellenberger and Schild, 2002). Later in the 1990s several groups simultaneously cloned a group of brain specific channels, which belong to the acid sensitive ion channel (ASIC) subfamily. The first sodium channel of this type to be cloned was initially named MDEG1, now renamed ASIC2 (also BNC1, for brain sodium channel 1, or BNaC1). and can be gated by protons (Price et al., 1996; Waldmann et al., 1996; Garcia-Anoveros et al., 1997; Waldmann et al., 1999). Furthermore, a mutation of the channel at the same position as that described to cause the death of cells expressing MEC-4d variant also caused activation of the ASIC2 (MDEG1) channel (Waldmann et al., 1996). At the moment the amiloride sensitive sodium channels responsive to protons have been renamed as acid sensitive ion channels, ASIC1-4 (Waldmann et al., 1999). Several members of the DEG/ENaC family of ion channels are expressed in dorsal root ganglion (DRG) neurons and as such might be candidate mechanosensitive ion channels in these cells. All the ASIC family members are prominently expressed in the DRG (Chen et al., 1998; Waldmann and Lazdunski, 1998), two of these channels ASIC3 (formerly known as DRASIC) and ASIC4 appear to be highly specifically expressed in DRG (Waldmann et al., 1997; Chen et al., 1998). However, it is not just members of the ASIC branch of the DEG/ENaC family that are expressed in the DRG, it has also been reported that $\alpha ENaC$, $\beta EnaC$, and $\gamma ENaC$ are also expressed by trigeminal sensory neurons (Fricke et al., 2000), the functional significance of such expression is yet to be determined.

The ASIC protein ASIC2, also named MDEG1, BNC1, and BNaC1 (Waldmann et al., 1996; Garcia-Anoveros et al., 1997; Price et al., 2000), appeared a promising candidate as a touch-receptor channel as its expression is enriched in large sensory neurons of the DRG (Price et al., 2000; McIlwrath et al., 2005). Electrophysiological recordings from single mechanoreceptor neurons from BNC1/ASIC2 knockout mice revealed that the sensitivity of RA neurons was substantially reduced in the absence of the ASIC2 subunit. The ASIC2a protein can make homomultimeric proton-gated channels when expressed in heterologous expression systems whereas its splice variant ASIC2b cannot (Lingueglia et al., 1997). In the BNC1/ASIC2 knockout mice both splice variants were deleted with the targeting strategy used (Price et al., 2000). As pointed out earlier it does not necessarily need to be the case that mechanotransduction channels form ligand-gated ion channels when expressed heterologously, as physiological gating may occur via force transmitted through attached extracellular matrix components. Even though the ASIC2a subunit is gated by low pH (Waldmann et al., 1996), the receptor endings of low-threshold mechanoreceptors, which express this channel, are not excited by low-pH solutions (Steen et al., 1992). One striking aspect of the BNC1/ASIC2 knockout phenotype is that only low-threshold mechanoreceptors, preferentially RA neurons, are affected in the mutant, although many more sensory neurons undoubtedly express the channel (Price et al., 2000; Garcia-Anoveros et al., 2001; Alvarez de la Rosa et al., 2002). It is clear from what is known of the DEG/ENaC superfamily of channels that heteromeric channels are the rule (Waldmann and Lazdunski, 1998; Kellenberger and Schild, 2002). In addition, all four of the ASICs are expressed in the DRG (ASIC1-4) (Waldmann and Lazdunski, 1998), thus it appears likely that loss of one subunit can change the subunit composition and perhaps kinetic properties of the channel complex (Benson et al., 2002). Again using the skin-nerve preparation, a detailed analysis was also undertaken of sensory neuron mechanosensitivity in ASIC3 (originally named DRASIC) knockout mice. Here RA mechanoreceptors were also dramatically altered, but this time they exhibited an *increased* sensitivity to moving stimuli (Price et al., 2001). We have confirmed this finding using a more sophisticated computer controlled mechanical stimulus. Since RA mechanoreceptors are exclusively responsive to a moving stimulus we tested the velocity coding of RA mechanoreceptors in ASIC3 mutant mice and also observed a twofold increase in the firing rate of mutant neurons (Moshourab and Lewin, unpublished). The MEC-4 ion channel is a pore-forming subunit of mechanotransducing elements in C. elegans paired body touch mechanoreceptors (Ernstrom and Chalfie, 2002). Functional studies strongly indicate that these neurons are also rapidly adapting movement detectors (Suzuki et al., 2003a; O'Hagan et al., 2005). We have argued that ASIC2 and ASIC3 are likely to form part of the mechanotransducing element of primary afferent mechanoreceptors (Price et al., 2000; Price et al., 2001). Since transduction is not abolished by deletion of either of these genes, it could still be argued that mechanoreception is altered because in the absence of ASIC proteins membrane excitability and therefore action potential generation is altered. So far no evidence has been found indicating such changes in ASIC mutant mice (Price et al., 2000; Benson et al., 2002; Drew et al., 2004). Some groups have started to investigate in detail mechanically gated conductances in the membrane of acutely isolated cultured DRG neurons (McCarter et al., 1999; Drew et al., 2002, 2004). Although, some heterogeneity in the kinetics of evoked currents was observed a clear correspondence with identified mechanoreceptor spike responses to natural stimuli found *in vivo* was not apparent. In one study, mechanically gated currents in sensory neurons from ASIC2, ASIC3, and ASIC2/3 double knockout mice were examined (Drew et al., 2004). No difference was observed in the mechanically gated currents from neurons between the different genotypes, and the authors concluded that these channels do not contribute to the mechanically gated current (Drew et al., 2004). After axotomy and ligation of peripheral nerves, mechanosensitivity is observed to appear after a delay consistent with fast axonal transport in the blind neuroma stump (Koschorke et al., 1991, 1994; Michaelis et al., 1999). It is therefore conceivable that transduction elements in axotomized neurons observed in culture are related to the in vivo situation. More data is obviously needed to determine if the mechanically gated conductance in cultured sensory neurons is a prerequisite for mechanosensitivity in vivo. Another member of the ASIC family that is highly expressed in the DRG is ASIC1. We have examined mechanosensory responses of a large number of mechanoreceptor types including nociceptors in ASIC1a knockout mice (Wemmie et al., 2002). We found no quantitative difference in the mechanosensitivity of any mechanoreceptor type in these mice indicating that this subunit is unlikely to participate directly in mechanotransduction in cutaneous sensory neurons (Page et al., 2004). However, the absence of the ASIC1 channel subunit did alter the mechanosensitivity of some visceral sensory neurons that detect stretch in the stomach and gastrointestinal tract (Page et al., 2004). The localization of the MEC-4 containing channels along C. elegans sensory axons is very punctate (Zhang et al., 2004). The punctate distribution of the channels might be controlled by extracellular matrix factors and might be necessary for the normal function of such channels (Emtage *et al.*, 2004; Zhang et al., 2004). We have shown that ASIC2 channels are also present in a puncta along the neurites of cultured DRG neurons (McIlwrath et al., 2005). The level of ASIC2a in DRG neurons is also controlled by BDNF availability in vivo (McIlwrath et al., 2005), this is interesting as the sensitivity of slowly adapting mechanoreceptors in BDNF deficient mice is substantially reduced (Carroll et al., 1998).

Another *mec* gene that is essential for mechanotransduction in *C. elegans* body touch neurons codes for a stomatin-like protein. Stomatin is an integral membrane protein that is thought to regulate ion channels (Stewart, 1997). Stomatin is expressed in virtually all mouse DRG neurons and may play a role in the transduction of mechanical stimuli (Mannsfeldt *et al.*, 1999). We have examined the mechanosensitivity of mechanoreceptors in the stomatin null mutant mice and found that only minor changes in the sensitivity of these neurons to mechanical stimuli could be observed (Mannsfeldt, Martinez-Salgado, and Lewin, unpublished results). Nevertheless, at least three other stomatin-like proteins are expressed by sensory neurons and at least one of these may be essential for sensory neuron mechanotransduction (Heppenstall, Wetzel, Mannsfeldt, and Lewin, unpublished).

IX. ARE TRP CHANNELS CANDIDATES FOR THE SENSORY NEURON MECHANOTRANSDUCER?

It is clear from the studies in *C. elegans* as well as in *Drosophila* (discussed earlier) that channels belonging to the TRP family are potentially very good candidates for the vertebrate sensory neuron mechanotransduction channel. The TRP channel family is very large and diverse and many of the TRPs have been directly implicated in sensory transduction events (Clapham et al., 2003; Corey, 2003). The prototypical sensory neuron and thermosensitive TRPV1 ion channel, first discovered as the receptor for the pungent compound capsaicin (Caterina et al., 1997), has a well-described role in the transduction of noxious heat and chemicals (Jordt et al., 2003). The detailed examination of knockout mice, where the TRPV1 gene was deleted, has not revealed a role for TRPV1 in the transduction of mechanical stimuli (Caterina et al., 2000). Membership of the TRPV (V stands for vanilloid) subfamily of TRPs is assigned on the basis of sequence similarity and there are three other members TRPV2, TRPV3, and TRPV4 that have been implicated in the detection of thermal stimuli (Patapoutian et al., 2003). Although, TRPV2 is certainly expressed by a small subpopulation of sensory neurons (Caterina et al., 1999), the expression of TRPV3 and TRPV4 in sensory neurons remains somewhat controversial (Liedtke et al., 2000; Peier et al., 2002; Smith et al., 2002; Xu et al., 2002; Suzuki et al., 2003c). The effects of deleting TRPV2 on mechanosensation have not yet been reported, but since this protein is expressed in such a small population of neurons, it is unlikely to play significant role in the majority of mechanoreceptors (Caterina et al., 1999; Woodbury et al., 2004). Two groups have generated a null mutation of the TRPV4 gene in mice (Liedtke and Friedman, 2003; Suzuki et al., 2003b). TRPV4 codes for a TRP ion channel that when expressed in heterologous cells can be activated by osmotic stimuli (Liedtke et al., 2000; Strotmann et al., 2000), and a very small number of sensory neurons might express significant amounts of the TRPV4 protein (Liedtke et al., 2000). The major site of expression for both TRPV3 and TRPV4 is in skin keratinocytes (Chung et al., 2004). The functional significance of such a site of expression is hard to gauge as so far no physiological studies exists that indicate that these cells are mechanosensitive and can transfer this information to the sensory fibers that innervate the epidermis. Nevertheless, it has been claimed by two groups that the loss of TRPV4 function leads to a significant increase in the behavioral threshold of the animals to noxious mechanical stimuli (Liedtke and Friedman, 2003; Suzuki et al., 2003b). In both cases direct recordings from primary afferent mechanoreceptors have not been made to determine if loss of TRPV4 function leads to a loss of mechanoreceptor sensitivity. The behavioral changes observed may be due to multiple factors associated with loss of TRPV4 function, for example, changes in spinal or supraspinal circuits that process somatosensory signals. The TRPV4 is the mammalian ortholog of the Osm-9 protein, a *C. elegans TRP* gene, which mediates osmotic avoidance and nose touch in ciliated sensory neurons. Liedtke and colleagues introduced the mammalian *TRPV4* into *C. elegans* nose sensory neurons and showed that using an appropriate promotor *TRPV4* could rescue the osmotic avoidance and nose touch behaviors associated with loss of Osm-9 function (Liedtke *et al.*, 2003).

The group of Uhtaek Oh has been able to observe stretch activated conductances in the membrane of rat DRG neurons both in whole-cell mode or from excised membrane patches (Cho et al., 2002). These channels were activated with a relatively long latency by membrane stretch (>1 s), which is an attribute not expected from a somatosensory transduction channel. Lowthreshold mechanoreceptors can fire spikes within just a few milliseconds of stimulus onset (Shin et al., 2003), and even nociceptors respond with spikes to a suprathreshold mechanical stimulus within 100 ms of stimulus onset (Moshourab, Milenkovic, and Lewin, unpublished observations). This does not mean that channels recorded by Oh and colleagues are not interesting candidates, as it is quite possible that the mode of mechanical gating is different than membrane stretch. In recent years several groups have started to characterize a mechanically activated current in cultivated DRG neurons (sometimes called I_{mech}). In these experiments the cell soma is normally stimulated directly with a mechanical probe, and a fast mechanically gated inward current is observed (McCarter et al., 1999; Drew et al., 2002, 2004). The assumption here is that the mechanically gated conductance represents the mechanically activated receptor potential normally present at the peripheral endings of the neurons in vivo. In at least one study it was found that the channels activated by these mechanical stimuli are sensitive to block by ruthenium red an agent known to be active on TRP channels. The molecular nature of the underlying conductance still remains unclear and there appears to be a large variation in the size and kinetics of the current measured by different groups. For example, one report has claimed that benzamil, an amiloride analog, is capable of blocking I_{mech} (McCarter *et al.*, 1999), however, another report found no amiloride sensitivity, but found that the current could be blocked by the TRP channel blocker ruthenium red (Drew et al., 2004). This latter finding at least suggests that a TRP channel might underlie I_{mech} . There are two other TRP channels that are not members of the TRPV subfamily and are also expressed by a substantial number of primary sensory neurons. These are the menthol sensitive channel TRPM8 and the mustard oil sensitive channel TRPA1 (formerly known as ANKTM1) (Story et al., 2003; Bandell et al., 2004; Jordt et al., 2004). So far gene knockout studies have not been published with these two channels,

and it is therefore not possible to directly evaluate their role in mechanotransduction. The TRPA1 channel is particularly interesting, as this channel is expressed by a large number of small diameter sensory neurons and the *Drosophila* ortholog of the *TRPA1* gene is *painless*, a TRP channel, required for normal mechanosensory and noxious heat behaviors in fly larvae (Tracey *et al.*, 2003). It should be noted that the *Drosophila* multidendritic neurons that express *painless* are not ciliated mechanosensory neurons.

Genetic screens in C. elegans and Drosophila have identified five genes encoding TRP channels, Ocr-4, Osm-9, NompC, Nanchung, and Inactive, whose activity is necessary for a variety of ciliated mechanosensory function. The hair cells of the inner ear are ciliated mechanoreceptors and here genetic screens in invertebrates have been successful at pointing toward candidate hair cell mechanotransducing channels. For example, a hearing mechanotransducing channel was identified in zebra fish by looking for orthologs of the NompC gene. NompC, the only TRP family member, implicated in bristle receptor mechanotransduction in Drosophila so far (Walker et al., 2000) has a zebra fish ortholog, which is expressed in sensory hair cells. A combination of computation and standard molecular biology techniques identified the NompC ortholog in zebra fish (Sidi et al., 2003) that has an overall domain structure identical to the Drosophila NompC, this includes conservation of the highly unusual 29 ankyrin repeats present in the Drosophila protein (Sidi et al., 2003). Using morpholinos to knockdown the expression of the zebra fish NompC, Sidi and colleagues were able to provide evidence that sensory hair cells require this protein to transduce sound. The comparison of C. elegans and Drosophila NompC with vertebrate genome databases and cDNA libraries has not uncovered other vertebrate NompC genes (Gillespie and Walker, 2001; Strassmaier and Gillespie, 2002). Sidi and colleagues were unable to find a homolog of zebra fish *NompC* in either the human or mouse databases (Sidi et al., 2003).

Expression and function of a candidate *TRP* gene in invertebrate ciliated mechanosensory neurons may not be a prerequisite for the function of the orthologous gene in hair cells. Very recently Corey and colleagues obtained evidence that the *TRPA1* gene might be candidate for the hair cell mechanotransduction channel both in mice and in zebra fish (Corey *et al.*, 2004). The data showing that TRPA1 might be a hair cell channel in both zebra fish and mouse was based on the use of small interfering mRNA (morpholinos or siRNA), it is interesting to note that the effects of TRPA1 morpholinos in zebra fish on the auditory response were similar to but not as extensive as those found with morpholinos directed against zebra fish NompC. This data suggest that the hair cell channel might consist of more than one TRP channel in zebra fish (Sidi *et al.*, 2003; Corey *et al.*, 2004), this has already

been suggested for the rudimentary hearing apparatus of *Drosophila* where both *Nanchung* and *Inactive* are required (Gong *et al.*, 2004).

In summary, the case for a direct involvement of a TRP channel in sensory neuron mechanotransduction can only be made by analogy to nonmammalian species. It remains to be seen whether any of the known *TRPs* that are expressed in sensory neurons are directly involved in the transduction of high- or low-intensity mechanical stimuli by nociceptive or nonnociceptive sensory neurons. More detailed electrophysiological work on mutant mice is required.

X. EXPRESSION CLONING

Another possible approach to find mechanotransduction components is to use expression cloning. This technique can be implemented in various forms but essentially requires the establishment of cellular assays whereby cDNAs can be expressed in cells and the function of the expressed protein tested. A classical version of this paradigm is to find genes that encode proteins that bind and are activated by a highly specific ligand. A very successful example of this approach is the cloning of the capsaicin receptor by identifying pools of cDNAs that when used to transfect kidney 293 cells induced a de novo capsaicin responsiveness identified with Fura based calcium imaging (Caterina et al., 1997). In this case the authors identified the first TRP protein with an essential role in the detection of chemical and possibly noxious thermal stimuli (Tominaga et al., 1998; Woodbury et al., 2004). One drawback of this approach is that it can usually only identify proteins whose function alone is sufficient by itself to reconstitute the activity assayed for. Thus, the capsaic binding activity of the TRPV1 channel has turned out to be a property of this channel and no other (Caterina et al., 2000). Since most known mechanosensory units are assembled from many different proteins, for example, at least nine components are part of the C. elegans mechanotransduction system, the use of expression cloning may be problematic in this case.

However, in some cases expression cloning has been used to identify molecules that are potentially part of the mechanotransducer. An early attempt to use expression cloning to identify mechanosensitive ion channels was made by Nakamura and Strittmatter (1996). Pools of cDNA clones derived from embryonic chick DRG were injected into *Xenopus* oocytes and found to confer a large inward current response to puff of buffer onto the oocyte. This activity was isolated to one single clone coding for the chicken P2Y1 receptor, a metabotropic ATP receptor. It was not shown in this paper that this receptor directly responds to membrane stretch but rather might respond to ATP released from the oocyte as a result of buffer puff (Nakamura and Strittmatter, 1996). The physiological significance of such a mechanism for fast mechanotransduction is still unclear, for instance, no touch insensitive phenotypes have been reported from the P2Y1 receptor knockout mouse (Fabre *et al.*, 1999). Furthermore, although single unit recording have been made from low-threshold mechanoreceptors in the presence of high concentrations of ATP no enhancement of mechanosensitivity was noted (Hamilton *et al.*, 2001).

In the case outlined above a mechanical stimulus applied to oocvtes was the phenotype screened for after injection of the cRNA. Some groups have hypothesized that the sensitivity to osmotic stress might be a feature of mechanosensitive channels (see section on TRP channels). The idea is that cell swelling might activate channels that are sensitive to membrane stretch. Some sensory neurons in culture do appear to respond to hypotonic solutions although the physiological significance of such a stimulus is not clear (Viana et al., 2001; Alessandri-Haber et al., 2003). Three TRP channels, TRPV4, Inactive, and Nanchung, have been implicated in mechanotransduction and can be opened with osmotic stress when expressed in a heterologous system (Liedtke et al., 2000; Strotmann et al., 2000; Kim et al., 2003; Gong et al., 2004). Caprini and colleagues, therefore, decided to use an expression cloning strategy to identify novel genes activated by osmotic stress. For their investigation they used a human DRG cDNA library to transfect human embryonic kidney cells (HEK 293) transiently. In a functional screen, they measured intracellular calcium concentration with the ratiometric indicator dye Fura-2, in particular, changes in calcium in response to a hypo-osmotic stimulus (30% hypotonic in contrast to isotonic solution). They were able to identify an activity in a pool of cDNA clones that dramatically enhanced the cells response to the hypo-osmotic stimulus. Iterative subdivision of the pool led to the identification of a single cDNA clone that mediated this effect. Surprisingly, the clone coded for a well-characterized phosphoprotein called growth-associated protein (GAP)-43 (also known as neuromodulin) (Caprini et al., 2003). GAP-43 is expressed in neuronal growth cones and highly upregulated during neuronal development and regeneration (Strittmatter, 1992). They further showed that membrane anchoring is essential for the protein to facilitate intacellular calcium rise in response to osmotic stress. GAP-43 is targeted to the membrane through palmitoylation of two cysteine residues and the protein also interacts with lipid rafts (Maekawa et al., 1997; Arni et al., 1998; Laux et al., 2000). Removal of the cysteine residues essential for membrane anchoring of GAP-43 also abolished the osmosensory activity of the protein.

The experiments by Caprini and colleagues showed that the intracellular calcium increase in response to somatic stress was due to release of calcium from intracellular stores. The experiments show that GAP-43 might be a

downstream transducer of changes of osmotic stress, but the experiments also indicate that HEK cells might be able to respond to osmotic stress via expression of endogenous osmosensitive proteins. The significance of GAP-43 for transduction of physiologically meaningful signals by sensory neurons has yet to be investigated. It was interesting that this approach failed to identify any DRG expressed cDNA species coding for integral membrane proteins or membrane ion channels like TRPV4.

XI. IMAGING MECHANOTRANSDUCTION WITH FLUORESCENT DYES

It has been known for some time that the styryl dye FM1-43 is an excellent tool to image the recycling of membrane vesicles (Betz and Bewick, 1992; Betz et al., 1996). This dye was shown some time ago to be able to pass through and simultaneously block mechanotransduction channels in sensory hair cells (Nishikawa and Sasaki, 1996; Gale et al., 2001). This observation was taken one step further by Meyer and colleagues (Meyers et al., 2003) who showed that not just sensory hair cells could be labeled with FM1-43 but many other types of sensory cells. Thus by injecting FM1-43 or its fixable analog AM1-43 systemically or locally, they could label sensory cells with this dye. They provided evidence that the dye gained access via the sensory terminal endings. It was not however, shown that this entry was via transduction channels. The fact that the dye did not enter central neurons was taken as evidence that the labeling was not simply via endocytic uptake (Meyers et al., 2003). Since FM1-43 labels cells via ion channels, and possibly sensory transduction ion channels, this is an obvious methodology to screen for genes that confer a mechanically induced entry of this dye into heterologous cells.

XII. USING DNA MICROARRAYS TO FIND TRANSDUCTION COMPONENTS

The DRG consists of a large variety of sensory mechanoreceptor types. It is reasonable to assume that the molecular and cellular mechanisms by which these different mechanoreceptors transduce mechanical signals might be different. Work on neurotrophin knockout mice has revealed that neurotrophic factors can have very specific survival effects on distinct classes of sensory receptors (Airaksinen *et al.*, 1996; Lewin, 1996; Carroll *et al.*, 1998; Minichiello *et al.*, 1998; Stucky *et al.*, 2002). With this in mind we have decided to use gene arrays to screen for differentially expressed genes in neurotrophin knockout mice. We decided to make use of the fact that the D-hair mechanoreceptor neuron is completely missing in mature mice in which the *neurotrophin-4* (*NT-4*) gene was inactivated (Stucky *et al.*, 1998, 2002). D-hair mechanoreceptors are probably the most sensitive vertebrate mechanoreceptors and are particularly sensitive to very slowly moving mechanical stimuli (Dubreuil *et al.*, 2004).

We decided to use the NT-4 knockout mice (NT- $4^{-/-}$) to try and identify genes specifically expressed in D-hair mechanoreceptors. Thus, we were looking for genes that are expressed in the DRG only in sensory neurons that are D-hair mechanoreceptors. Such a gene might be expressed in other cell types or tissues, but in the DRG its expression must be specific to D-hair mechanoreceptors. In order to find such genes we used the fact that NT-4 knockout mice lose D-hair receptor neurons only after 5 weeks of age (Stucky et al., 2002). D-hair receptors make up only around 6% of all DRG neurons. Thus, genes of interest should be expressed only in a small number of DRG neurons in young (<5 weeks old) NT-4 knockout or wildtype mice and would be absent in adult NT-4 knockout mice. To look for such genes we used affymetrix oligonucleotide microarrays of more than 10,000 genes expressed around 189 genes, which had a reduced expression in adult NT-4 knockout mice compared to young NT-4 knockout mice and controls. To try and narrow down the list of possible candidates we also used the cDNA suppression subtractive hybridization (SSH) technique (Diatchenko et al., 1996) for the generation of a subtracted cDNA library. With this approach, Shin and colleagues were able to reduce the number of candidate genes obtained from the DNA microarrays experiments down to 28. To verify the results and to further confine the number of genes, in situ hybridization experiments were carried out (Fig. 5). Of the 28 genes identified only 22 were detectable in DRG neurons with in situ hybridization and just 12 genes were confirmed as downregulated with real-time PCR. But not all of these downregulated genes showed the required enrichment in a subpopulation of sensory neurons. Only three of the identified genes appeared to be expressed in a subpopulation of DRG neurons, these were the shaker-related potassium channel K_y 1.1, the T-type calcium channel Ca_y3.2, and Trk B the receptor for BDNF. Only two of these putative marker genes, Ca_x3.2, and Trk B, fulfilled the criteria for D-hair specificity as DRG neurons expressing these genes disappear in adult NT-4 knockout mice (Stucky et al., 2002; Shin et al., 2003). The fact that TrkB is expressed at a high level in D-hair receptor neurons is not entirely surprising as this is also the cellular receptor for NT-4. Thus, when D-hair mechanoreceptors acquire an absolute dependence on NT-4 after 5 weeks of age, cells expressing the NT-4 receptor also die (Stucky et al., 2002). The most interesting finding was that D-hair mechanoreceptors express very high amounts of the low voltagegated T-type calcium channel Ca_v3.2. This calcium channel belongs to a



FIGURE 5 In situ hybridization experimenst in sections of the mouse DRG to identify putative D-hair mechanoreceptor markers. A probe directed against $K_v 1.1$, a voltage-gated potassium channel reveals strong expression in a subset of medium and large diameter neurons (A) in wild-type DRG. Examination $K_v 1.1$ expression in NT-4 mutant did not reveal any dramatic loss of $K_v 1.1$ positive neurons (B), thus this ion channel is unlikely to be expressed exclusively by D-hair receptor neurons. In panels C–E in situ hybridization results with a specific probe against Ca_v3.2. Ca_v3.2 is expressed in a subset of medium sized DRG neurons in WT mice (C) and the number of Ca_v3.2 positive neurons is slightly reduced in young NT-4 knockout mice (D) but is dramatically reduced in old NT-4 knockout mice (E). The quantification of Ca_v3.2 positive neurons in WT-4 knockout mice is also shown (F) (3 animals per group). ** indicates significant difference p < 0.001 unpaired *t*-test. Positive neurons were counted as a proportion of all the unstained neurons with a clear nucleus viewed under phase contrast. This figure is modified from Fig. 1 Shin *et al.* (2003).

subfamily of three other genes that encode T-type calcium channels, but the other T-type channels were barely detectable in the DRG and were not regulated in a manner coincident with the loss of D-hair mechanoreceptors. We have obtained evidence using pharmacological blockade of the T-type calcium channel that this channel might contribute to the extremely high sensitivity of D-hair mechanoreceptors (Shin et al., 2003; Dubreuil et al., 2004). We hypothesize that because this channel is activated very near to the resting membrane potential, the small receptor potentials produced by very small stimuli will activate the channel. We know that D-hair mechanoreceptors can respond to mechanical displacements of as little as 1 µm (unpublished observations). The finding that D-hair mechanoreceptors express very high amounts of Ca_y3.2 suggested an explanation for an observation made many years before, namely, that a small number of sensory neurons in culture exhibit very (several nanoamps in amplitude) large T-type calcium currents (Schroeder et al., 1990). We directly showed that D-hair mechanoreceptors with a unique morphology in culture exhibit large T-type calcium currents, and these neurons are also dependent on NT-4 in culture (Fig. 6). Electrophysiological studies from several groups have shown that also small diameter sensory neurons, which are probably nociceptors, express a T-type calcium current, although the amplitude of such currents is around tenfold smaller than those expressed in D-hair receptors (Todorovic et al., 2001). It has been found that use of relatively selective T-type calcium antagonists can be effective analgesics, especially for neuropathic pain (Todorovic et al., 2002; Dogrul et al., 2003; Todorovic et al., 2004). This is surprising in light of one report that T-type calcium channels are downregulated in nociceptive sensory neurons following neuropathic nerve injury (McCallum et al., 2003). The effectiveness of targeting T-type calcium channels has been extended with the use of oligonucleotide knockdown experiments (Bourinet et al., 2004). In these experiments the authors explicitly claim that it is knockdown of the $Ca_v 3.2$ channel in sensory neurons and not in the spinal cord, where T-type calcium channels have been reported in nocireceptive laminae, which is responsible for the reversal of neuropathic symptoms in treated rats (Bourinet et al., 2004). This is an odd finding since we found that pharmacological blockade with low doses of the T-type calcium antagonist Mibefradil did not have any effect on the mechanosensitivity of both A-fiber and C-fiber nociceptors under normal circumstances, however, the mechanosensitivity of D-hair mechanoreceptors was substantially inhibited (Shin et al., 2003). One alternative model to explain these results is that $Ca_v 3.2$ is essential for the normal function of D-hair afferents and it is the activation of these neurons that directly leads to allodynia. Thus the idea would be that the central connectivity of D-hair mechanoreceptors changes after nerve injury so as to activate spinal circuits leading to pain (Lewin et al., 2004).



FIGURE 6 Morphological and functional identification of D-hair neurons *in vitro*. (A) Neurofilament staining of a neuron with a rosette like morphology induced by incubation with 10 ng/ml NT-4. Scale bar 40 µm. (B) The percentage of rosette neurons was calculated relative to the total neuronal population and was significantly reduced in NT-4^{-/-} mutant mice. (C) A ramp protocol applied to a rosette neuron from -80 mV to +40 mV, 500 ms duration with the whole-cell patch clamp technique showed the presence of a high-amplitude low voltage-activated I_{Ca} . This calcium current profile was observed exclusively in the rosette like neurons. (D) Dose–response curve of NiCl₂ inhibition of low voltage-activated I_{Ca} . The inhibitory effect of Ni was evaluated on calcium currents elicited with ramp protocols. Dose response curve was fitted with a Hill equation $y = V_{\text{max}} \{\text{Ni}\}^n / (\text{K}_{1/2} + \{\text{Ni}\})^n$ (dashed line). From fit of the data, the Ni²⁺ concentration for half-maximal effect K_{1/2} was 2.9 ± 0.2 µM. Reproduced with permission from Dubreuil *et al.* (2004), **24**, 8480–8484.

This particular model has yet to be tested, but could be tested with the use of NT-4 or $Ca_v 3.2$ knockout mice.

XIII. BIOCHEMICAL APPROACHES

Classically transduction components have been biochemically purified from sensory organs. For example, this approach proved to be a feasible

13. Finding Sensory Neuron Mechanotransduction Components

technique for the purification of transduction components from retinal photoreceptors. Since the density of transduction components within photoreceptors is extremely high and one can isolate literally millions of photoreceptors from a single retina biochemical purification proved highly successful. In contrast, the peripheral endings of sensory endings are the only part of the neuron (Fig. 2) that is mechanosensitive in vivo and these very small endings (Fig. 1) are embedded in a huge mass of skin tissue. However, it is possible to gather very large amounts of skin tissue even from one animal in contrast to the sensory epithelia that contain hair cells in the inner ear. With this in mind we have been experimenting with techniques to biochemically separate or enrich for fractions of the skin that contain sensory nerve endings. The idea behind our approach was that the small size of nerve endings in the skin might allow them to be separated by the density vescicular fraction. Skin tissue isolated from neonatal mice was minced and the vesicles were separated according to their density using a sucrose gradient. Fractions were collected and sometimes further treated with methyl- β -cyclodextrin an agent that can solubilize raft proteins by sequestering membrane cholesterol.

In order to determine if such a biochemical enrichment strategy is feasible, we performed western blotting using antibodies against sensory neuron markers. Our preliminary data suggests that it might be possible to biochemically isolate membrane fractions from the skins, which are enriched in transduction components. It is conceivable that this methodology could be combined with other techniques in order to prepare and purify transduction proteins for mass spectrometry and sequencing. For example, it has become possible to screen for differentially expressed peptides or proteins using methodologies such as Isotope coded affinity tags (ICAT) (Gygi et al., 1999; Freeman and Hemby, 2004; Zhong et al., 2004). The principle of this method is to take two protein mixtures and label each either with a light or heavy ICAT reagent. The heavy ICAT reagent can chemically modify proteins by reacting with thiol groups (cysteines) and consists of a biotin tag with a linker in which hydrogens have been replaced with deuterium-the light ICAT reagent has only hydrogen groups. Two solubilized protein mixtures are treated with the light and heavy ICAT reagents and labeled peptides are affinity purified after protein digestion using the ICAT biotin tag. Peptides that are enriched in one sample compared to the other will be visualized in the mass spectrometer as the peptides from each sample will co-elute, but there will be an 8 Da mass difference measured in a scanning mass spectrometer. Peptides that appear to be enriched in one sample can then be taken further into the tandem mass spectrometer to identify the peptide and searching of the available databases should in most cases uniquely identify the protein from which this peptide is derived. The advantage of such a technique is that small and highly complex protein mixtures can be compared. One can envisage several scenarios in which such a technique could be used to isolate putative sensory transduction components. For example, biochemical isolates from denervated and intact skin might reveal novel proteins localized in sensory endings.

XIV. CONCLUSIONS

In this chapter we have tried to review the current state of knowledge concerning the identity of sensory mechanotransduction components. We have put particular emphasis on the experimental strategies that have been used to try and identify mechanotransduction channels and the associated proteins. It is clear that candidate gene approaches based on the results from noninvertebrates can be very useful in identifying potential transduction components. The data from genetic screens in other organisms also provides a conceptual model to work with to try and guide experiments. It seems likely for instance that mechanotransduction components in the endings of sensory neurons are present in multimolecular complexes that may be difficult to characterize in their native state. With further advances in our knowledge of mechanotransduction components linked with the rapid progress in the availability of proteomic tools might in future make the direct elucidation of the mechanosensor feasible.

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CHAPTER 14

Functional Diversity of Voltage-Dependent Ca²⁺ Channels in Nociception: Recent Progress in Genetic Studies

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I. OVERVIEW

Voltage-dependent Ca^{2+} channels (VDCCs) play a pivotal role in sensory processing, including nociception, by increasing the intracellular Ca^{2+} concentration of neurons in response to excitation. Pain-related behaviors in several VDCC knockout mice have been studied to yield significant progress in elucidating the physiological importance of various subtypes of VDCCs in pain transmission at the level of the organism. We review here recent studies defining the behavioral consequences in mice lacking diverse subunits of VDCCs in response to various types of painful stimuli and compare these phenotypes with data obtained from pharmacology-based approaches. These collective studies from pharmacological and genetic approaches reveal that selective modulations of different VDCCs may provide novel therapeutic modalities for treatment of various types of pain.

II. INTRODUCTION

Sensory stimuli are relayed from the periphery to the cortex as a form of action potential via a series of excitation of relay neurons (Adrian, 1928, 1959). VDCCs play a pivotal role in sensory transduction by increasing the intracellular Ca^{2+} concentration of neurons in response to excitation. VDCCs are also involved in the regulation of neurotransmitter release, membrane excitability, electrical spiking behavior, and gene expression (Huguenard, 1996; Miller, 1998, 2001).

Mediators of pain and inflammation, such as bradykinin, serotonin, and histamine, trigger the release of nociceptive neurotransmitters, including glutamate, substance P, and calcitonin gene-related peptide (CGRP), from nerve terminals of primary afferent neurons and contribute to the sensitization of spinal processing of pain signals (Millan, 1999). L-, N-, and P/Q-type calcium channels are involved in the release of pain-related neurotransmitters (Rane *et al.*, 1987; Maggi *et al.*, 1990; Vedder and Otten, 1991; Pfrieger *et al.*, 1992; Santicioli *et al.*, 1992; Takahashi and Momiyama, 1993; Turner *et al.*, 1993; Gruner and Silva, 1994; Evans *et al.*, 1996). Accordingly, a physiological role for VDCCs in pain perception was proposed (Millan, 1999). Consistent with this theory, various VDCC blockers are effective in the control of different pain states (Hernandez *et al.*, 1993; Abdulla and Smith, 1997; Sluka, 1997, 1998; Vanegas and Schaible, 2000; Matthews and Dickenson, 2001; Knight *et al.*, 2002; Todorovic *et al.*, 2002, 2004a,b; Fukuizumi *et al.*, 2003; Heinke *et al.*, 2004).

Studies involving the characterization of molecular targets for VDCCdependent pain control have also utilized subtype-selective drugs. However, owing to limited selectivity and specificity, the effects of VDCC antagonists on nociception vary depending on the drug, dosage, and route of administration. Thus, more effective drugs and techniques are required to determine the precise roles of various VDCCs in nociception. Gene-knockout technologies (Fig. 1) have been added to the repertoire of conventional methods to produce significant progress in elucidating the physiological importance of VDCCs in pain transmission *in vivo* at the level of the gene (Saegusa *et al.*, 2000, 2001; Hatakeyama *et al.*, 2001; Kim *et al.*, 2001a,b, 2003; Murakami *et al.*, 2002; Clark *et al.*, 2003).



FIGURE 1 Schematic diagram for producing knockout mice.

In this review, we describe the behavioral consequences of specific VDCC mutations in mice in response to various types of pain stimuli (Table I) and compare these phenotypes with data obtained from a range of pharmacology-based approaches.

III. VDCC FAMILY

VDCCs are divided into two major classes according to the membrane potential at which they are activated, specifically, high voltage-dependent (HVD) Ca^{2+} channels and low voltage-dependent (LVD) Ca^{2+} channels, otherwise known as T-type Ca^{2+} channels (Tsien *et al.*, 1991, 1988; Miller, 2001; Perez-Reyes, 2003). The HVD channels are further divided into L-, N-, P/Q-, and R-type, which are distinguished by their voltage dependence, kinetics, and pharmacology.

	Nociceptive response												
Null gene	Μ	lechan	ical		Therma	ıl		Inflam	nation		Neuro	pathic	
(subunit, channel type)	vF	ТР	TPr	HP	PW	TF	F-P1	F-P2	AC	CFA	NI-m	NI-t	References
Ca _v 1.3 (α_{1D} , L-type)	_	_		_									Clark et al. (2003)
$Ca_v 2.2^a$ (α_{1B} , N-type)	\downarrow	\downarrow	\downarrow	_	\downarrow	\downarrow	_	\downarrow	\downarrow				Kim et al. (2001a)
Cav2.2 ^b (a1B, N-type)	_			_	_	\downarrow	_	\downarrow	_		\downarrow	\downarrow	Saegusa et al. (2001)
$Ca_v 2.2^c$ (α_{1B} , N-type)		_		\downarrow			_	\downarrow					Hatakeyama et al. (2001)
Ca _v 2.3 (α_{1E} , R-type)	_	_		_	_	_	_	_	_				Saegusa et al. (2000)
Ca _v 3.1 (α_{1G} , T-type)	_	_		_	_	_			Ť	-			Kim et al. (2003)
$Ca_v\beta 3 \ (\beta 3)$	_			\downarrow		\downarrow	_	\downarrow		_			Murakami et al. (2002)
Leaner (tg^{la}/tg^{la}) (\alpha_{1A}, P/Q-type)	\downarrow			Î									Ogasawara et al. (2001)

 TABLE I

 Nociceptive Responses in KO Mice for Calcium Channel Function

Nociceptive assay: AC, abdominal constriction (writhing) test; CFA, complete Freund's adjuvant hyperalgesia; F-P1, formalin test, phase 1; F-P2, formalin test, phase 2; HP, hot-plate test; NI-m, mechanical allodynia following nerve injury; NI-t, thermal hyperalgesia following nerve injury; PW, paw-withdrawal; TF, tail-flick; TPr, tail pressure; TP, tail-pinch; vF, von Frey fiber mechanical sensitivity. Symbols: \uparrow , KOs more sensitive than WTs; – , no difference between genotypes; \downarrow , KOs less sensitive than WTs.



FIGURE 2 Structural organization of voltage-gated Ca²⁺ channels.

The VDCC structure consists of pore-forming α_1 , auxiliary β , $\alpha_2\delta$, and, in some cases, γ subunits (Hofmann *et al.*, 1999; Catterall, 2000; Kang *et al.*, 2001) (Fig. 2). The α_1 subunit is essential for channel functions, including selectivity, permeation, and voltage sensing, and is the determinant for pharmacological typing of VDCCs, specifically, L- (α_{1S} , α_{1C} , α_{1D} , and α_{1F}), P/Q- (α_{1A}), N- (α_{1B}), R- (α_{1E}), and T- (α_{1G} , α_{1H} , and α_{1I}) type (Tsien *et al.*, 1991, 1988; Miller, 2001; Perez-Reyes, 2003). Auxiliary subunits modulate the trafficking and biophysical properties of the α_1 subunits (Arikkath and Campbell, 2003; Klugbauer *et al.*, 2003). Currently, VDCC classification is based on sequence analysis of subunit genes. Ten genes encode for α_1 , four for β , four for $\alpha_2\delta$, and eight for γ subunits (Ertel *et al.*, 2000) (Table II).

The pharmacological and genetic diversity of VDCCs and differential expression patterns of their subtypes in the peripheral and central nervous systems (CNS) (Table III) contribute to the functional heterogeneity of these channels in the modulation of pain signals.

Current type	Subunit name (Gene name)	Alternative name	Associated subunits	Tissue distribution	Blockers
	α_l subunits				
L	α_{1S} (<i>Cacnals</i>)	Ca _v 1.1	$\alpha_2\delta, \beta, \gamma$	Skeletal muscle	Verapamil, diltia- zem, dihydropyri- dine, nipedipine
	α_{1C} (<i>Cacna1c</i>)	Ca _v 1.2	$\alpha_2\delta,\beta,\gamma$	Heart, brain, pancreas	
	α_{1D} (<i>Cacna1d</i>)	Ca _v 1.3	$\alpha_2\delta,\beta,\gamma$	Brain, pancreas	
	α_{1F} (<i>Cacna1f</i>)	Ca _v 1.4	n.e.	Retina	n.e.
P/Q	α_{1A} (<i>Cacna1a</i>)	Ca _v 2.1	$\alpha_2\delta, \beta 1, 3, 4,$ possibly γ	Brain, heart, pancreas	ω-agatoxin IVA, ω-conotoxin MVIIC
Ν	α_{1B} (<i>Cacna1b</i>)	Ca _v 2.2	$\alpha_2\delta, \beta, 3, 4,$ possibly γ	Brain	 ω-conotoxin GVIA, MVIIC, ω-cono- toxin MVIIA (SNX-111)
R	α_{1E} (<i>Cacnale</i>)	Ca _v 2.3	$\alpha_2\delta, \beta,$ possibly γ	Brain	SNX-482
Т	α_{1G} (<i>Cacnalg</i>)	Ca _v 3.1	$\alpha_2\delta$	Brain, heart, placenta	Mibefradil, kurtoxin, nickel
	α_{1H} (<i>Cacna1h</i>)	Ca _v 3.2	n.e.	Brain, heart, kidney	
	α ₁₁ (<i>Cacna1i</i>) Auxiliary subunits	Ca _v 3.3	n.e.	Brain	
	$\alpha_2 \delta_{1-4}$ (Cacna2d ₁₋₄)	$Ca_v \alpha 2\delta$		Brain, heart, skeletal muscle	
	$\beta_{1-4} (Cacnb_{1-4})$	$Ca_{v}\beta$		Brain, heart, lung, skeletal muscle	
	$\gamma_{1-8} (Cacng_{1-8})$	$Ca_v\gamma$		Brain, heart, lung, skeletal muscle	

 TABLE II

 Classification of Voltage-Dependent Ca²⁺ Channels and Auxiliary Subunits

n.e., not established.

			Pain Si	gnaling		
	Subunit	Expres	sion pattern in related to nocic	brain regions ception		
Current type	alternative name	DRG	Spinal cord	Supraspinal region	References	
	α_l subunits					
L	$\alpha_{\rm lD}, {\rm Ca_v 1.3}$	S, M, L	Dorsal horn		Scroggs et al. (1992); Acosta and Lopez (1999)	
P/Q	$\alpha_{lA}, Ca_v 2.1$	S, M, L	Dorsal horn (laminae II-VI)		Scroggs et al. (1992); Acosta and Lopez (1999)	
N	α_{lB} , Ca _v 2.2	S, M, L	Dorsal horn (laminae I-II)	PAG, RVM	Scroggs <i>et al.</i> (1992); Acosta and Lopez (1999); Saegusa <i>et al.</i> (2001)	
R	$\alpha_{IE}, Ca_v 2.3$	S, M, L	Dorsal horn	RVM	Saegusa et al. (2000)	
Т	$\alpha_{lG}, Ca_v 3.1$	n.d.	Dorsal horn (laminae I-II)	PAG, thalamus, spinal TGN	Scroggs et al. (1992); Acosta and Lopez (1999)	
	$\alpha_{\rm lH},Ca_v 3.2$	S, M	Dorsal horn (laminae I)	PAG, thalamus	Scroggs et al. (1992); Talley et al. (1999)	
	α ₁₁ , Ca _v 3.3 Auxiliary subunit	S, M	Dorsal horn			
	$\alpha_2\delta$, $Ca_v\alpha_2\delta$	S, M, L	Dorsal horn		Luo et al. (2001)	
	β 3, Ca _v β 3	S, M, L	Dorsal horn			

TABLE III

Expression Patterns of Voltage-Dependent Calcium Channels in Areas Related to Pain Signaling

S, small-size neurons; M, medium-size neurons; L, large-size neurons; DRG, dorsal root ganglion; PAG, periaqueductral gray; RVM, rostral ventromedial medula; TGN, trigerminal nuclei; n.d., not determined.

A. L-Type Ca²⁺ Channels in Nociception

L-type Ca^{2+} channels are implicated in nociception, both in the spinal cord (Ryu and Randic, 1990) and DRG neurons (Wyatt *et al.*, 1997; Acosta and Lopez, 1999). In DRG neurons, nifedipine, a blocker of voltage-dependent L-type Ca^{2+} channels, completely inhibits the potassium-induced

release of substance P, which is triggered by mediator substances of pain and inflammation, such as bradykinin, serotonin, and histamine (Rane *et al.*, 1987; Vedder and Otten, 1991), indicating that L-type calcium channels are involved in the release of nociceptive neurotransmitters.

To date, pharmaco-behavioral studies using L-type antagonists have disclosed conflicting results on acute pain responses to noxious stimuli. On the one hand, intrathecal administration of L-type channel blockers, verapamil and nifedipine, had no effect on pain response in several different behavioral assays, including the hot-plate test (Malmberg and Yaksh, 1994), mechanical stimuli, or noxious heat to the hindpaw in normal rats (Sluka, 1997, 1998). On the other hand, intraperitoneal injection of verapamil reduced mechanical and thermal pain in rats (Todorovic et al., 2004b) and mice (Weizman *et al.*, 1999). These discrepancies signify that the effects of L-type Ca²⁺ channel antagonists on nociception vary depending on the type of drug, dosage, route of administration, and test methods employed (Prado, 2001). Clark and colleagues (2003) examined acute pain responses to noxious stimuli in mice lacking the α_{1D} gene. No differences in mechanical and thermal pain thresholds were evident between the α_{1D} mutant and wild-type mice. The results suggest that the α_{1D} channel is not required for acute pain perception or alternatively reflect developmental compensation (Clark et al., 2003) such as upregulation of α_{1C} in $\alpha_{1D}^{-/-}$ mice (Namkung *et al.*, 2001). Thus, other L-type channels might be involved in acute pain signaling.

B. N-Type Ca²⁺ Channels

N-type Ca²⁺ channels mediate synaptic transmission in the mammalian CNS (Maggi *et al.*, 1990; Santicioli *et al.*, 1992; Gruner and Silva, 1994). These channels are present in acutely dissociated small sensory neurons of DRG (Scroggs and Fox, 1992; Abdulla and Smith, 1998; Acosta and Lopez, 1999; Honma *et al.*, 1999). Activation of N-type channels triggers the release of pain signal-related neurotransmitters, such as glutamate, substance P, and CGRP, from primary afferent terminals in the dorsal horn of the spinal cord (Maggi *et al.*, 1990; Santicioli *et al.*, 1992; Gruner and Silva, 1994; Evans *et al.*, 1996), implying that these channels are therapeutic targets for pain control.

1. N-Type Ca²⁺ Channels in Acute Pain

Studies on the function of N-type Ca^{2+} channels in nociception initially focused on acute pain responses mediated by interactions between peripheral sensory neurons and neurons in the dorsal horn of the spinal cord. The current results from studies using blockers to investigate the function of

N-type calcium channels in acute nociception in response to mechanical or thermal stimuli are ambiguous. Blocking N-type calcium channels had little or no effect on acute pain responses. Sluka (1997) observed no change in response to mechanical stimulation of the paw when ω -conotoxin GVIA (ω -CTxGVIA), a specific blocker of N-type Ca²⁺ channel isolated from the snail *Conus geographu*, was administered intrathecally to the spinal cord (for blockers see Table I). Moreover, Sluka (1998) and Wang *et al.* (2000) detected no effect on paw withdrawal responses to heat, following injection of SNX-111 (ω -conotoxin MVIIA, another N-type Ca²⁺ channel blocker) to the spinal cord via a microdialysis fiber. On the other hand, intrathecal injection of SNX-111 in awake rats increased latency in the hot-plate test (Malmberg and Yaksh, 1994). Thus, pharmacological studies performed to date have failed to clarify the precise role of N-type channels in acute pain transmission.

Mice lacking the α_{1B} subunit of the N-type Ca²⁺ channel have been independently generated by three different groups and examined for nociceptive responses (Hatakeyama et al., 2001; Kim et al., 2001a; Saegusa et al., 2001). The $\alpha_{1B}^{-/-}$ mice from all three groups displayed complete absence of N-type Ca^{2+} currents in sensory neurons, with no accompanying modulation of other channels. However, the three groups reported distinct results from behavioral assays. For example, in experiments by Kim et al. (2001a) α_{1B} -deficient mice displayed reduced responses to noxious mechanical stimuli delivered on the paw and tail by von Frey filaments, while the other two groups reported no difference between α_{1B}^{-7-} and wild-type mice in these assays (Hatakeyama *et al.*, 2001; Saegusa *et al.*, 2001). In addition, $\alpha_{1B}^{-/-}$ mice displayed prolonged latency of response to thermal stimuli evoked by radiant heat on the palm skin or tail (Kim et al., 2001a; Saegusa et al., 2001), indicating that a null mutation of the N-type Ca²⁺ channel reduces the spinal reflexes mediated by the A δ fiber (Chapman *et al.*, 1985). Hatakeyama *et al.* (2001) reported reduced responses in $\alpha_{1B}^{-/-}$ mice with the hot-plate test (another thermal pain paradigm), while the other two groups observed no difference in similar experiments (Kim et al., 2001a; Saegusa et al., 2001). The different genetic backgrounds of mice and variations in the testing environments or test procedures used possibly underlie these discrepancies.

2. N-Type Ca²⁺ Channels in Inflammatory Pain

Different chemical substances, such as formalin, adjuvant, and capsaicin, which induce inflammation, elicit various pain behaviors on peripheral application. The N-type Ca^{2+} channel is involved in central sensitization in the spinal cord induced by intradermal injection of chemicals. The importance of the N-type calcium channel in pain perception has been confirmed in several models of inflammation. Intrathecal N-type channel blockers

reduce hyperalgesia initiated by knee joint inflammation (Neugebauer *et al.*, 1996; Sluka, 1998) or intraplantar injection of capsaicin (Sluka, 1997). In particular, in the formalin test, intrathecal injection of SNX-111 or ω -CTxGVIA is effective in controlling pain perception (Malmberg and Yaksh, 1994, 1995; Bowersox *et al.*, 1996; Diaz and Dickenson, 1997).

In earlier experiments, ω -CTxGVIA suppressed phase 2 and less efficiently, phase 1 pain responses in the formalin test (Malmberg and Yaksh, 1994, 1995; Diaz and Dickenson, 1997). Bowersox *et al.* (1996) showed that a lower dose of SNX-111 was partially effective in inhibiting phase 1 pain responses, while the maximally effective dose prevented both phases almost completely. In contrast to the variable findings attained with N-type channel blockers, consistent results were obtained with α_{1B} mutant mice from all three groups in the formalin test. The $\alpha_{1B}^{-/-}$ mice displayed reduced responses only in phase 2, with normal phase 1 responses. The onset of phase 2 was significantly delayed, and the duration of response was shorter in mutant mice, compared to their wild-type counterparts (Hatakeyama *et al.*, 2001; Kim *et al.*, 2001a; Saegusa *et al.*, 2001).

Moreover, acute administration of SNX-111 induced motor problems, such as whole body shaking, circling, ataxia, and tail wiggling, in wild-type mice (Bowersox *et al.*, 1996), which was not observed in $\alpha_{1B}^{-/-}$ animals. These results suggest that N-type channel blockers have side effects as a result of nonspecific binding to other channels. An alternative explanation is that in the mutant, some of the potential problems of N-type deletion are developmentally compensated for by an undefined mechanism. Thus, data from pharmacological and genetic studies collectively indicate that N-type Ca²⁺ channels play a major role in the processing of inflammatory pain signals associated with central sensitization.

3. N-Type Ca²⁺ Channels in Neuropathic Pain

Peripheral nerve injury causes a spontaneous persistent pain, designated as neuropathic pain, measured by the potentiation of mechanical allodynia and thermal hyperalgesia in animals (Kim and Chung, 1992).

Saegusa and colleagues (2001) reported that $\alpha_{1B}^{-/-}$ mice display markedly reduced development of mechanical allodynia and thermal hyperalgesia, which are induced by spinal nerve injuries. These results are in accordance with data obtained from experiments involving intrathecal administration of SNX-111 (Bowersox *et al.*, 1996; Yamamoto and Sakashita, 1998). N-type Ca²⁺ channels are evidently implicated in the expression of neuropathic pain, suggesting that blockers of these channels are potential therapeutic agents for pain suppression.

SNX-111 is highly effective in suppressing inflammatory and neuropathic pain in animal models (Bowersox *et al.*, 1996). However, dose-dependent

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adverse effects, such as dizziness, blurred vision, nystagmus, and sedation, are observed in human studies. In rats, higher doses of this agent result in severe motor problems, including whole body shaking, circling, ataxia, and tail waggling. The side effects may not be due to the suppression of N-type Ca^{2+} channels, since $\alpha_{1B}^{-/-}$ mice do not exhibit these motor problems, although developmental compensation in the mutant cannot be ruled out as the cause of this discrepancy. This knockout mouse is a valuable tool for characterizing the origins of diverse pharmacological effects or side effects of SNX-111 (Eglen *et al.*, 1999).

C. P/Q-Type Ca²⁺ Channels in Nociception

In addition to N-type channels, P/Q-type channels play essential roles in the release of neurotransmitters, such as glutamate, serotonin, norepinephrine, GABA, and glycine, in the CNS including the spinal cord (Pfrieger *et al.*, 1992; Takahashi and Momiyama, 1993; Turner *et al.*, 1993). However, the importance of Ca^{2+} influx through P/Q-type channels in physiological and pathological pain states remains to be elucidated.

Data from pharmacological analyses indicate a minimal role of P/Q-type Ca^{2+} channels in nociception. Injection of ω -agatoxinIVA (ω -AgaIVA) to the spinal dorsal horn has no effect on pain responses to either mechanical (Sluka, 1997) or heat stimuli (Sluka, 1998). Additionally, the drug exerts no significant analgesic effects in the hot-plate test or chronic neuropathic pain models (Chaplan *et al.*, 1994). However, intrathecal injection of ω -AgaIVA strongly suppresses phase 2 pain responses in the formalin test, suggesting that P/Q-type Ca²⁺ channels participate in pain transmission at the spinal level (Malmberg and Yaksh, 1994).

At present, there are no convenient mouse models for P/Q-type Ca²⁺ channel deficiency to use in pain sensitivity assays, since null mutant mice for the α_{1A} gene have severe motor problems, low survival rate, and absence seizures, making it difficult to use these animals for behavioral assays (Jun *et al.*, 1999; Fletcher *et al.*, 2001; Song *et al.*, 2004). Nevertheless, Ogasawara *et al.* (2001) attempted to measure pain responses by using pups (P7–9) of *leaner* (tg^{la}/tg^{la}) mice (a spontaneous P/Q-type Ca²⁺ channel mutant of a weak allele) before their motor problems became severe. *Leaner* mice displayed reduced response to mechanical stimuli but increased response to noxious thermal stimuli. The investigators explained enhanced thermal sensitivity with reduced dorsal root potential. Dorsal root potential is derived from modest depolarization of the terminals of primary afferent fibers by inhibitory interneurons (Rudomin and Schmidt, 1999; Willis, 1999). It was proposed that the P/Q-type channel is preferentially involved in inhibitory

interneurons that modestly depolarize primary afferent terminals, leading to the suppression of neurotransmitter release from primary afferent terminals. However, the issue of whether the P/Q-type calcium channel is involved in GABA release to primary afferent terminals is currently unclear. In summary, in contrast to the results obtained with pharmacological studies, behavioral studies using *leaner* mice suggest that P/Q-type channels function in acute mechanical pain transmission in the spinal dorsal horn.

D. R-Type Ca²⁺ Channels in Antinociception

R-type Ca²⁺ currents are heterogeneous mixtures of several different currents *resistant* to blockers for L-, N-, P-, and Q-type Ca²⁺ channels (Zhang *et al.*, 1993), also known as *residual* or *remained* currents. The α_{1E} subunit contributes significantly to R-type Ca²⁺ currents (Wilson *et al.*, 2000; Lee *et al.*, 2002). Based on the finding that the α_{1E} gene is expressed in DRG and the dorsal horn of the spinal cord (Saegusa *et al.*, 2000; Wilson *et al.*, 2000; Yusaf *et al.*, 2001), it is proposed that the R-type Ca²⁺ currents play an important role in pain signal transmission. However, Saegusa *et al.* (2000) reported that $\alpha_{1E}^{-/-}$ mice displayed similar responses to their wild-type counterparts in the paw-flick, tail-flick, and hot-plate tests.

The investigators mentioned previously additionally examined the involvement of R-type Ca²⁺ channels in descending antinociceptive pathways, since α_{1E} is expressed in periaqueductal gray (PAG). The PAG region is involved in the descending antinociceptive mechanism to control the excitability of spinal neurons. A preceding experience of visceral pain activates a long-lasting descending antinociceptive mechanism, which reduces behavioral responses to painful stimuli delivered at a later stage (Kurihara *et al.*, 2003). $\alpha_{1E}^{-/-}$ mice lacked such anitinociceptive mechanisms (Saegusa *et al.*, 2000). Thus, α_{1E} channels provide a useful tool for analyzing the mechanism of the descending antinociceptive pathway.

E. T-*Type* Ca²⁺ Channels

1. T-Type Ca²⁺ Channels in Acute Pain

A number of studies have disclosed that T-type Ca^{2+} channels play a crucial role in pronociceptive mechanisms such as boosting pain signals at the periphery or activity-dependent sensitization of signals in the spinal cord (Carbone and Lux, 1984; Todorovic *et al.*, 2001, 2002; Ikeda *et al.*, 2003; Heinke *et al.*, 2004). For example, a systemic injection of mibefradil, a preferential T-type Ca^{2+} channel blocker, induces an antinociceptive effect



FIGURE 3 Boosting of pain signals by T-type Ca²⁺ channels in the periphery.

against noxious mechanical or thermal stimuli (Todorovic *et al.*, 2002). Todorovic *et al.* (2001) additionally showed that the T-type Ca²⁺ channel blocker injected locally inhibits redox agent-induced thermal hyperalgesia in adult rats. The group proposed that T-type Ca²⁺ channels in the periphery serve as amplifiers of nociceptive transmission. In contrast, Kim and colleagues (2003) demonstrated that mice lacking α_{1G} T-type Ca²⁺ channels displayed normal responses to cutaneous mechanical or thermal nociceptive stimuli, supporting the theory that α_{1G} T-type Ca²⁺ channels are not involved in peripheral nociceptive mechanisms. It is feasible that the pronociceptive function of T-type Ca²⁺ channels observed at the periphery in blocker experiments is mediated by other subtypes of T-type Ca²⁺ channels such as α_{1H} and/or α_{1I} (Fig. 3).

2. T-Type Ca²⁺ Channels in Inflammatory Pain

 α_{1G} -deficient mice displayed stronger writhing responses to visceral pain stimuli than wild-type mice (Kim, *et al.* 2003). Following injection into the peritoneum, peripherally acting mibefradil caused analgesia, whereas transient focal infusion of mibefradil into the thalamus enhanced behavioral responses to visceral pain. As shown in Fig. 4, deletion of α_{1G} T-type Ca²⁺ channels obliterated thalamic burst firing (Kim *et al.*, 2001b), which may



FIGURE 4 Intrinsic firing properties of TC neurons located at ventrobasal complex. (A) The presence (wild type) or absence (mutant) of burst firing patterns elicited by 100 ms of negative step-current inputs at -70 mV. (B) The presence (wild type) or absence (mutant) of burst firing patterns elicited by positive step-current inputs at -80 mV. Only the high-frequency spikes are missing in $\alpha_{1G}^{-/-}$ TC neurons. (C) Tonic firing patterns elicited by positive step-current inputs at -60 mV. (D) The relation between the number of spikes and the amount of current injected. The number of spikes during 100 ms positive step-current inputs either when membrane potentials are held at -60 mV (left) or at -80 mV (right). Reprinted from *Neuron* (2001) Jul 19, **31**(1), 35–45.

reduce and/or inhibit relay of information to the cortex (Steriade *et al.*, 1993). According to a report, nociceptive information from viscera is relayed to the cortex through thalamocortical (TC) neurons of the ventroposterolateral (VPL) region of the thalamus (Willis *et al.*, 1999). Thus, hyperalgesia to visceral pain in α_{1G} -deficient mice may be explained by a lack of the inhibitory mechanism of visceral pain exerted by burst firing at the thalamic level.

To further investigate the mechanism of thalamic control of pain by T-type Ca²⁺ channels, the firing patterns of TC neurons in VPL regions were analyzed using single-unit recordings (Fig. 5). Visceral pain evoked earlyonset single spikes and gradually increased burst firings in responsive neurons in the VPL region in wild-type animals. As the frequency of burst firings increased, that of single spikes was dramatically reduced (Fig. 5A and B, middle). In α_{1G} -deficient mice, early onset increase of single spikes was observed as in the wild-type animals, but the negligible burst activity of VPL neurons remained unaltered, following acetic acid injection (Fig. 5A and B, bottom). In the absence of significant burst activity, pain-induced single spike responses persisted in TC neurons of $\alpha_{1G}^{-/-}$ mice. These results suggest that α_{1G} T-type Ca²⁺ channels in the thalamus play an inhibitory role in the processing of persistent nociceptive signals, thereby suppressing pain responses. Based on the data, we propose that α_{1G} T-type Ca²⁺ channels mediate a novel thalamic control mechanism of persistent pain signals (Fig. 6). The opposing roles of T-type Ca^{2+} channels in pain signaling in the periphery versus brain are summarized in Fig. 7.

3. T-Type Ca²⁺ Channels in Neuropathic Pain

A number of studies suggest that T-type currents induce neuronal hyperexcitability of primary sensory neurons by lowering the threshold for action potentials and promoting bursting activity (Carbone and Lux, 1984; Huguenard, 1996; Amir *et al.*, 2002). Systemic administration of mibefradil or ethosuximide effectively reverses the behavioral manifestation of neuropathic pain in the spinal nerve ligation model (Dogrul *et al.*, 2003). The pharmacological modulation of peripheral T-type channels alters nerve injury-induced thermal hyperalgesia (Todorovic *et al.*, 2004a). The data support the hypothesis that peripheral T-type calcium channels are involved in neuropathic pain expression.

In addition to the peripheral mechanism, central hyperactive states resulting from the alteration of plasticity at synapses between nociceptive afferents and spinal dorsal horn neurons contribute to the pathogenesis of neuropathic pain (Mayer *et al.*, 1999; Woolf and Salter, 2000; Hunt and Mantyh, 2001). Ikeda and colleagues (2003) demonstrated that T-type calcium channels are involved in the induction of long-term potentiation (LTP) at these synapses. These results support the theory that T-type calcium channels are implicated in the development and maintenance of neuropathic pain. A separate study additionally shows that T-type channels modulate the responses of spinal dorsal horn neurons that play a crucial role in the development and maintenance of neuropathic pain (Matthews and Dickenson, 2001). These



FIGURE 5 Firing pattern changes in VPL neurons triggered by visceral pain in wild-type and $\alpha_{1G}^{-/-}$ mice. (A) Sample tracings obtained from the three experimental groups (+/+, saline; +/+, acetic acid; -/-, acetic acid) are shown for three periods. (B) The temporal change of the firing patterns. (C) Baseline firing rates obtained from results collected for 5 min preceding the acetic acid injection. Total, burst, and single firing rates in Hz are compared for the two genotypes. Two-tailed *t* test, **p* < 0.001; ***p* > 0.05. (D) Prolonged single-spike response in $\alpha_{1G}^{-/-}$ VPL neurons to visceral pain. Repeated ANOVA for genotype (post hoc test), **p* < 0.05; ***p* > 0.1. Reprinted from *Science* (2003) Oct 3, **302**(5642), 117–119.

findings support a potential role of T-channels in neuropathic pain expression. No genetic studies on T-type Ca²⁺ channels in neuropathic pain have been reported. Mice lacking the α_{1G} or α_{1H} T-type Ca²⁺ channels (Kim *et al.*, 2001b, 2003; Chen *et al.*, 2003) should be useful tools in clarifying this issue.



FIGURE 6 Antinociceptive function of α_{1G} T-type Ca²⁺ channels in the thalamus.



FIGURE 7 Differential roles of T-type Ca²⁺ channels in pain processing.

F. Auxiliary Subunits of Voltage-Dependent Ca²⁺ Channels

1. $\alpha_2 \delta$ Subunits in the Development of Allodynia

To date, four genetically distinct $\alpha_2\delta$ subunits ($\alpha_2\delta$ -1 $-\alpha_2\delta$ -4) have been described (Arikkath and Campbell, 2003; Klugbauer *et al.*, 2003). These $\alpha_2\delta$ subunits modify the various biophysical and pharmacological properties of the α_1 subunit. $\alpha_2\delta$ -1 subunit modulates membrane trafficking of α_1 , increases in current amplitude, and regulates activation and inactivation

kinetics. Unlike $\alpha_2\delta$ -1, $\alpha_2\delta$ -2 subunit increases the current amplitude, with no significant alteration in the biophysical properties. $\alpha_2\delta$ -3 subunit increases in current density, modulates voltage dependence of activation, and shifts the steady sate activation/inactivation curves. $\alpha_2\delta$ -4 subunit is the most recently described. The function of $\alpha_2\delta$ -4 subunit in the modulation of biophysical properties remains to be determined. Among these, $\alpha_2\delta$ -1 is the most extensively studied for its role in the development of neuropathic pain (Field *et al.*, 2000; Luo, 2000; Luo *et al.*, 2001, 2002). Previous studies confirm that gabapentin, an effective drug for neuropathic pain (Hwang and Yaksh, 1997; Abdi *et al.*, 1998), selectively interacts with the $\alpha_2\delta$ -1 subunit *in vitro* (Gee *et al.*, 1996). In addition, nerve injuries lead to increased $\alpha_2\delta$ -1 expression in the spinal cord and DRG, consistent with the idea that this subunit plays a role in allodynia, a term for enhanced nociceptive responses to innocuous stimuli (Luo, 2000; Luo *et al.*, 2001, 2002). However, genetic analyses utilizing mutant animal models are yet to be performed.

2. β_3 Subunit in Pain Processing

The β subunit aids in the trafficking of α_1 to the plasma membrane and modulates the biophysical properties of the channels with characteristics specific to the α_1 - β combination. In contrast to $\alpha_2\delta$ subunits, a positive function of the β subunit in pain perception has been established by an investigation using gene-targeting methods (Murakami *et al.*, 2002). Mice lacking β_3 of VDCCs displayed dramatically reduced pain responses elicited by acute thermal or persistent inflammatory noxious stimuli, suggesting a role of this subunit in ascending pain pathways of the DRG and spinal cord. An important function of β_3 in pain processing is closely related to N-type and P/Q-type Ca²⁺ channels, since this subunit mainly associates with α_{1B} and α_{1A} subunits (Scott *et al.*, 1996; Ludwig *et al.*, 1997; Moreno *et al.*, 1997). The decreased pain susceptibility of $\beta_3^{-/-}$ mice is consistent with the antinociceptive actions of N- or P/Q-type Ca²⁺ channel blockers (Malmberg and Yaksh, 1994, 1995; Diaz and Dickenson, 1997) and attenuated pain responses of $\alpha_{1B}^{-/-}$ mice (Hatakeyama *et al.*, 2001; Kim *et al.*, 2001b; Saegusa *et al.*, 2001).

IV. CONCLUSIONS

The functional diversity of VDCCs and limited selectivity of blockers for VDCC subtypes highlight the necessity for genetic studies, which allow the ablation of a specific gene for each subunit in the genome. Knockout mice, as well as spontaneous mutant lines, have significantly enhanced our understanding of the subtype-specific roles of VDCCs in the nociceptive

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mechanisms. A combination of pharmacological approaches with specific blockers and genetic tools using mice lacking specific VDCCs yields critical information about the role of each VDCC in specific types of pain signaling. Furthermore, such studies should aid in characterizing the potential side effects of specific channel blockers in adult animals. This information is critical in defining the therapeutic potentials of subtype-selective modulators of VDCC for the control of various types of pain.

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CHAPTER 15

Expression Patterns and Histological Aspects of TRP Channels in Sensory Neurons

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I. OVERVIEW

The molecular candidates for thermoreceptors of primary afferents have been clarified. These include six members of the transient receptor potential (TRP) superfamily of nonselective cation channels; TRPV1, TRPV2, TRPV3 (also known as VRL3), TRPV4 (also known as VRL-2/OTRPC4/ VR-OAC/TRP12), TRPM8 (also known as CMR1), and TRPA1 (renamed from ANKTM1). In this chapter, we summarize the distribution of these molecules in sensory neurons, especially among neuronal subpopulations of the dorsal root ganglion (DRG) and briefly discuss the functional meaning of their coexpression with other neuronal markers.

II. TRPV1 (VR1)

TRPV1 was cloned as the capsaicin receptor from a rat DRG cDNA library (Caterina et al., 1997). Since this channel also can be activated by noxious heat exceeding a threshold of 43°C, which causes pain in vivo (Tominaga et al., 1998), TRPV1 has been proposed as the first cloned thermal transducer. The overall distribution of TRPV1 transcript and protein does not appear to be as restricted to sensory ganglia as was suggested by early studies. Some regions in the central nervous system, as well as the epithelial cells of the skin and bladder, also express this receptor (Mezey et al., 2000; Birder et al., 2001; Denda et al., 2001). In the sensory nervous system, TRPV1 is expressed by not only the somatosensory neurons in the DRG, trigeminal ganglion (TG), and jugular ganglion (Ichikawa and Sugimoto, 2003) but also in visceral sensory neurons in the nodose ganglion (Ichikawa and Sugimoto, 2003). The most precise examination of the distribution of the TRPV1 among neuronal subpopulation was performed in the rat DRG (Table I). In this ganglion, about 40-60% of all neurons expressed the TRPV1, where most of such neuronal cell bodies were small or medium in size. They were rarely (3–4%) immunostained for 200 kDa neurofilament (Michael and Priestley, 1999), suggesting that TRPV1 is almost exclusively expressed by neurons with unmyelinated C-fibers (Ma, 2002). For the C-fiber neurons, although there exist a considerable overlap and exceptions, two major subpopulations have been proposed; the peptidergic and nonpeptidergic classes (Snider and McMahon, 1998). The former class has TrkA, a tyrosine kinase receptor specific for nerve growth factor (NGF), contains neuropeptides, such as substance P (SP) and calcitonin gene-related peptide (CGRP), and its central axons terminate in lamina I and the outer part of lamina II of the spinal dorsal horn. The significance of SP and CGRP in pain processing is well known, and the contribution of these neurons to sensory transmission is widely accepted. The latter class lacks TrkA and the aforementioned neuropeptides, is labeled by the Griffonia simplicifolia isolectin B4 (IB4), and its central axons terminate in the inner region of lamina II of the dorsal horn (Snider and McMahon, 1998). Rat TRPV1 is expressed by

TABLE I

The Percentages of TRPV-Positive Neurons and Coexpression with Other Molecules in Various Ganglia from a Variety of Species

	Reference
1. Rat DRG neurons (adult)	
TRPV1 mRNA/total = 46.7%	Michael and Priestley (1999)
TRPV1-ir(+)/total = 42% (culture)	Ahluwalia et al. (2000)
TRPV1-ir(+)/total = 58%	Guo <i>et al.</i> (2001)
TRPV1-ir(+)/total = 32-39%	Carlton and Hargett (2002)
TRPV1 mRNA/NF200-ir($-$) = 83.2%	Michael and Priestley (1999)
NF200-ir(-)/TVPV1 mRNA = 96.5%	Michael and Priestley (1999)
TRPV1-ir(+)/NF200-ir(-) > 40%	Ma (2002)
NF200-ir(-)/TRPV1-ir(+) = 30%	Ma (2002)
TRPV1 mRNA/TrkA-ir(+) = 65.2%	Michael and Priestley (1999)
TRPV1-ir(+)/SP-ir(+) = 85%	Tominaga et al. (1998)
TRPV1-ir(+)/SP-ir(+) = 77%	Guo et al. (1999)
SP-ir(+)/TRPV1-ir(+) = 33%	Guo et al. (1999)
TRPV1 mRNA/CGRP-ir(+) = 58.7%	Michael and Priestley (1999)
TRPV1-ir(+)/CGRP-ir(+) = 59%	Guo et al. (1999)
CGRP-ir(+)/TRPV1-ir(+) = 79%	Guo et al. (1999)
TRPV1-ir(+)/IB4-labeled = 60-80%	Tominaga et al. (1998)
TRPV1 mRNA/IB4-labeled = 74.9%	Michael and Priestley (1999)
TRPV1-ir(+)/IB4-labeled = 67%	Guo et al. (1999)
IB4-labeled/TRPV1-ir(+) = 78%	Guo et al. (1999)
TRPV1-ir(+)/P2X3-ir(+) = 75%	Guo et al. (1999)
P2X3-ir(+)/TRPV1-ir(+) = 75%	Guo et al. (1999)
TRPV1 mRNA/P2X3 mRNA = 59%	Kobayashi et al. (2004)
P2X3 mRNA/TRPV1 mRNA = 90%	Kobayashi et al. (2004)
TRPV1-ir(+)/CB1-ir(+) = 82% (culture)	Ahluwalia et al. (2000)
CB1-ir(+)/TRPV1-ir(+) = 98% (culture)	Ahluwalia et al. (2000)
TRPV1-ir(+)/CaMKII α -ir(+) = 38%	Carlton and Hargett (2002)
$CaMKII\alpha$ -ir(+)/TRPV1-ir(+) = 35-42%	Carlton and Hargett (2002)
TRPV1-ir(+)/SSTR2a-ir(+) = 60%	Carlton et al. (2004)
SSTR2a-ir(+)/TRPV1-ir(+) = 33%	Carlton et al. (2004)
2. Mouse DRG neurons	
TRPV1-ir(+)/total = 22.3%	Zwick <i>et al.</i> (2002)
TRPV1-ir(+)/IB4-labeled = 2%	Zwick et al. (2002)
IB4-labeled/TRPV1-ir(+) = 2.8%	Zwick et al. (2002)
3. Human TG neurons	
TRPV1-ir(+)/total neurons = 16%	Hou et al. (2002)
$CGRP-ir(+)/TRPV1-ir(+) = \sim 10\%$	Hou et al. (2002)
SP-ir(+)/TRPV1-ir(+) = 8%	Hou et al. (2002)
NOS-ir(+)/TRPV1-ir(+) = 5%	Hou et al. (2002)
	· · · ·

(Continued)

TABLE I (<i>Continued</i>)
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	Reference
4. Rat TG neurons	
TRPV1-ir(+)/total = 19.9%	Ichikawa and Sugimoto (2001)
TRPV1-ir(+)/P2X3-ir(+) = 34.7%	Ichikawa and Sugimoto (2004)
P2X3-ir(+)/TRPV1-ir(+) = 39.8%	Ichikawa and Sugimoto (2004)
5. Rat nodose ganglion neurons	
TRPV1-ir(+)/TRPV2-ir(+) = 63.4%	Ichikawa and Sugimoto (2003)
TRPV2-ir(+)/TRPV1-ir(+) = 3.2%	Ichikawa and Sugimoto (2003)
6. Rat jugular ganglion neurons	
TRPV1-ir(+)/TRPV2-ir(+) = 3.9%	Ichikawa and Sugimoto (2003)
TRPV2-ir(+)/TRPV1-ir(+) = 0.6%	Ichikawa and Sugimoto (2003)

Abbreviations: DRG, dorsal root ganglion; TG, trigeminal ganglion; mRNA, positive neurons obtained by *in situ* hybridization histochemistry; -ir(+), positive neurons obtained by immunohistochemistry; -ir(-), negative neurons obtained by immunohistochemistry; NF200, 200 kD neurofilament; SP, substance P; CGRP, calcitonin gene-related peptide; IB4, the *Griffonia simplicifolia* isolectin B4; CB1, cannabinoid receptor 1; CaMKII α , the α isoform of calcium-calmodulin-dependent protein kinase II; STR2a, somatostatin receptor 2a; NOS, nitric oxide synthase.

both of these classes (Tominaga *et al.*, 1998; Michael and Priestley, 1999; Guo *et al.*, 2001), and its colocalization with other molecules underlies many physiological phenomena as mentioned in the later sections.

A. TRPV1 and TrkA

A significant subpopulation of TrkA-expressing neurons coexpresses TRPV1 (Table I). TrkA receptor activation by NGF recruits multiple intracellular signaling pathways, which can bind to the intracellular phosphorylated tyrosine residues within TrkA. Three proteins in particular have been proposed as substrates of this kinase activity; Shc, phospholipase C γ 1, and phosphatidylinositol-3-kinase (PI3K) (Obermeier *et al.*, 1993; Dikic *et al.*, 1995). NGF acutely sensitizes the response of rat DRG neurons to capsaicin (Shu and Mendell, 1999). This sensitization mechanism seems to involve the early intracellular signaling pathway mediated byPI3K and later protein kinase C (PKC) and calcium-calmodulin-dependent protein kinase II (CaM-KII) in primary culture of mouse DRG neurons (Bonnington and McNaughton, 2003). The colocalization of TRPV1 and the α isoform of CaMKII has been reported in rat DRG (Carlton and Hargett, 2002) (Table I). The involvement of the extracellular signal-regulated protein kinase (ERK) pathway following PI3K activation has also been demonstrated in NGFinduced TRPV1 sensitization in rats (Zhuang *et al.*, 2004). On the other hand, chronic NGF upregulates TRPV1 expression and increases the proportion of capsaicin-sensitive DRG neurons in rats (Nicholas *et al.*, 1999; Ji *et al.*, 2002). In this case, another signal transduction cascade of NGF, the Shc-induced Ras/MEK pathway, seems to play key roles in the NGFinduced TRPV1 upregulation (Ji *et al.*, 2002; Bron *et al.*, 2003).

B. TRPV1 and SP

SP is contained within a limited subpopulation (20-30%) of small- to medium-size DRG neurons. These neurons have unmyelinated C-fibers or thinly myelinated A δ fibers (McCarthy and Lawson, 1989), and their central axons terminate in lamina I and the outer zone of lamina II of the spinal dorsal horn (Barber *et al.*, 1979; Gibson *et al.*, 1981; Hunt *et al.*, 1981; DiFiglia *et al.*, 1982; de Lanerolle and LaMotte, 1983). SP is released with glutamate following noxious stimulation and binds to the neurokinin 1 (NK1) receptor, one of the three G-protein–coupled receptors that recognize neurokinins. Activation of this receptor can induce the phosphorylation of the NMDA receptor, which is known to induce central sensitization of spinal dorsal horn neurons (Woolf and Salter, 2000). In this activation process, NK1 receptors rapidly desensitize and are subsequently internalized (Bowden *et al.*, 1994; Garland *et al.*, 1994; Mantyh *et al.*, 1995).

About 80% of SP-immunoreactive (ir) DRG neurons coexpress TRPV1 (Table I). TRPV1-ir primary afferents make monosynaptic contact with NK1 receptor-positive neurons in lamina I of the spinal dorsal horn (Hwang *et al.*, 2003), and capsaicin induces NK1 receptor internalization in rat spinal cord slices (Lao *et al.*, 2003). Noxious heat applied to peripheral tissues also induces SP release (Duggan *et al.*, 1987; McCarson and Goldstein, 1991) and NK1 receptor internalization in the spinal dorsal horn (Mantyh *et al.*, 1995; Abbadie *et al.*, 1997; Allen *et al.*, 1997; Liu *et al.*, 1997; Cao *et al.*, 1998; Riley *et al.*, 2001). And more importantly, the threshold for NK1 receptor internalization (43°C) has been demonstrated to be similar to the activation threshold for TRPV1 (Allen *et al.*, 1997).

C. TRPV1 and CGRP

CGRP is expressed by 45–70% of DRG neurons. About 60% of CGRP-ir neurons express TRPV1 in the rat DRG (Table I). In fact, capsaicin stimulates CGRP release from the sciatic nerve and the primary afferent terminals in the rat spinal cord at least partially via TRPV1 (Sauer *et al.*, 1999; Garry *et al.*, 2000). Noxious heat stimulation induces CGRP release from desheathed rat sciatic nerves with a threshold between 40°C and 42°C, similar to the activation threshold for TRPV1. However, heat-activated ion channels other than TRPV1 and TRPV2 may also be involved in this phenomenon (Sauer *et al.*, 2001).

D. TRPV1 and P2X Receptors

The ionotropic purine receptors, P2X receptors, are composed of an assembly of multiple P2X subunits. At present, seven subunits have been cloned and named "P2X1–7." Among them, much attention has focused on the P2X3, since this subunit is expressed almost exclusively in primary afferent neurons (Chen *et al.*, 1995) and the electrophysiological properties of the homomeric P2X3 receptor is similar to that of a native P2X receptor in the DRG (Chapter 11). P2X3 is expressed by 30–64% of all DRG neurons (Bradbury *et al.*, 1998; Vulchanova *et al.*, 1998; Fukuoka *et al.*, 2002; Kobayashi *et al.*, 2004). Almost all DRG neurons expressing TRPV1 coexpress P2X3 (Fig. 1) (Guo *et al.*, 1999; Ueno *et al.*, 1999; Kobayashi *et al.*, 2004). Therefore, capsaicin-sensitive afferents are usually ATP sensitive, while



FIGURE 1 Coexpression of TRPV1 and P2X3 mRNAs in rat DRG neurons. Bright-field photomicrographs of serial sections hybridized with radiolabeled probes for the TRPV1 (A) and P2X3 (B), and visualized by autoradiography. Arrows indicate the neurons expressing both channels.

a significant proportion of ATP-sensitive neurons are capsaicin insensitive (Ueno *et al.*, 1999; Petruska *et al.*, 2000).

E. TRPV1 and CB1 Receptor

Anandamide is an endogenous agonist to the cannabinoid 1 (CB1) receptor (Devane *et al.*, 1992) and also an agonist at the TRPV1. CB1 receptor activation by anandamide reduces neuronal excitability and transmitter release via inhibition of adenylate cyclase and voltage-gated Ca²⁺ channels, and activating inwardly rectifying K⁺ channels, which reduces CGRP release from primary afferents in the skin and spinal dorsal horn (Richardson *et al.*, 1998a,b). In contrast, high concentrations of anandamide induce CGRP release from primary afferents in peripheral tissue through activation of the TRPV1 (Zygmunt *et al.*, 1999). Surprisingly, the CB1 receptor and TRPV1 are well colocalized in primary culture of adult rat DRG neurons (Ahluwalia *et al.*, 2000) (Table I). On the other hand, it has been suggested that anandamide may sensitize TRPV1 via PKC activation, although the receptor that mediates this activation is unclear (Premkumar and Ahern, 2000).

F. TRPV1 and PKC

PKC activation directly activates and/or sensitizes the TRPV1 (Premkumar and Ahern, 2000; Numazaki *et al.*, 2002). Bradykinin and ATP induce and/ or enhance TRPV1 activity in a PKC-dependent manner via the B2 bradykinin receptor (Sugiura *et al.*, 2002) and P2Y receptors (Tominaga *et al.*, 2001), respectively. These receptors colocalize with TRPV1 to some extent in rat DRG neurons (Shin *et al.*, 2002; Moriyama *et al.*, 2003).

G. TRPV1 and PKA

Activation of cyclic AMP (cAMP)-dependent protein kinase (PKA) prevents TRPV1 desensitization through phosphorylation of the channel in primary afferent neurons (Bhave *et al.*, 2002). Prostaglandins, such as PGE2, produce hyperalgesia by raising intracellular cAMP levels and activating PKA in nociceptive afferents (Taiwo *et al.*, 1989; Taiwo and Levine, 1991). Conversely, mu-opioid agonists mediate peripheral analgesia by diminishing cAMP levels (Levine and Taiwo, 1989). However, the contribution of PKA in the rapid sensitization of TRPV1 appears unlikely (Bonnington and McNaughton, 2003).

H. TRPV1 and Somatostatin Receptor (SSTRs)

TRPV1 and SSTRa, one of the SSTRs, coexist in a subpopulation of rat DRG neurons (Table I), and activation of SSTRs modulates capsaicininduced TRPV1 activity and behavior (Carlton *et al.*, 2004).

III. TRPV2 (VRL1)

NF200-ir(+)/TRPV2-ir(+) = 81%

TRPV2 has been cloned as a homolog of the TRPV1 from the rat brain cDNA library (Caterina et al., 1999). This receptor is activated by high temperatures with a threshold of 52°C and is insensitive to capsaicin or acid. TRPV2 transcript is rather widely distributed among rat tissues such as spinal cord, brain, gut, lung, and spleen (Caterina et al., 1999). In the rat DRG, most TRPV2-ir neurons are medium to large in size and are immunostained by an antineurofilament antibody, RT97, suggesting that A-fiber neurons express this receptor (Table II) (Caterina et al., 1999; Ma, 2001). On the other hand, about one-third of TRPV2-ir neurons contained CGRP, while TRPV2-ir neurons were rarely IB4-positive or immunostained for TRPV1 or SP (Table II) (Caterina et al., 1999; Lewinter et al., 2004). These histochemical data and other electrophysiological studies (McCarthy and Lawson, 1997; Nagy and Rang, 1999) suggest that TRPV2 is preferentially expressed by $A\delta$ fiber neurons and may underlie the heat-responsive character of type I A δ mechano- and heat-sensitive neurons that are known to have a response threshold of 53°C (Meyer et al., 1994).

	(,,
TRPV2-ir(+)/total = 16%	
IB4-labeled/TRPV2-ir(+) = $\sim 2\%$	
$\text{SP-ir}(+)/\text{TRPV2-ir}(+) = \sim 5\%$	
CGRP-ir(+)/TRPV2-ir(+) = 36%	

 TABLE II

 TRPV2 Distribution Among Rat DRG Neurons (Caterina et al., 1999)

IV. TRPV3 (ALSO KNOWN AS VRL3)

Two groups have independently cloned human TRPV3 from brain and small intestine/colon cDNAs (Smith *et al.*, 2002; Xu *et al.*, 2002). They found high TRPV3 expression in the central nervous system, spinal cord, DRG, skin, and testis and lower expression in skeletal muscle, stomach, trachea, intestine, adipose tissue, and placenta (Smith *et al.*, 2002). An *in situ* hybridization histochemistry study with an antisense RNA probe for human TRPV3 revealed that most monkey DRG neurons of all sizes expressed TRPV3 mRNA (Smith *et al.*, 2002), while an immunohistochemistry study found only limited staining in a population of human DRG neurons (Xu *et al.*, 2002). Mouse TRPV3 was cloned from newborn skin cDNA, and TRPV3 mRNA was detected by PCR, but not by Northern blot analysis, in adult DRG tissue (Peier *et al.*, 2002b).

The TRPV1 and TRPV3 are coexpressed by the human DRG neurons, and these two receptors are associated with each other *in vitro*, suggesting the possibility of the formation of heteromeric TRPV channels (Smith *et al.*, 2002). TRPV1 (Denda *et al.*, 2001) and TRPV3 (Peier *et al.*, 2002b) are also expressed by keratinocytes of the skin. The TRPV3 is readily activated at body temperature, because its activation threshold is \sim 33–39°C (Smith *et al.*, 2002; Peier *et al.*, 2002b; Xu *et al.*, 2002). Taken together, TRPV3 may modulate the sensitivity of the TRPV1 by forming heteromeric TRPV channels in the skin as well as in the DRG.

V. TRPV4 (ALSO KNOWN AS VRL-2/OTRPC4/VR-OAC/TRP12)

TRPV4 was originally identified as an osmoreceptor (Liedtke *et al.*, 2000; Strotmann *et al.*, 2000; Wissenbach *et al.*, 2000; Delany *et al.*, 2001) and was later recognized as a heat receptor activated by warm temperatures with a threshold of \sim 27–32°C (Guler *et al.*, 2002; Watanabe *et al.*, 2002; Chung *et al.*, 2003). Although both Northern blot analysis and RT-PCR suggest the expression of TRPV4 mRNA in the DRG and TG of rats, no specific TRPV4 immunostain was observed in the cell bodies of these ganglia (Guler *et al.*, 2002). However, *in situ* hybridization histochemistry revealed TRPV4 mRNA is expression in a subpopulation of sensory neurons in the mouse TG (Liedtke *et al.*, 2000). The controversies over the expression and the precise distribution of this receptor in sensory neurons are open to study. Just like TRPV3, TRPV4 is also expressed in skin epidermal keratinocytes and mediate thermosensation in the warm range (Guler *et al.*, 2002; Chung *et al.*, 2003, 2004).

	X			
	TRPM8 (Peier et al., 2002a) (%)	TRPA1 (Story et al., 2003) (%)		
x mRNA/total	5–10	3.6		
x mRNA/NF150-ir(+)	0	0		
TRPV1-ir(+)/x mRNA	0			
TRPV1 mRNA/x mRNA		97		
x mRNA/TRPV1-ir(+)	0			
x mRNA/TRPV1 mRNA		30		
CGRP-ir(+)/x mRNA	0	97		
x mRNA/IB4-labeling	0			

 TABLE III

 Comparative Distribution of Cold-Activated Channels in the Adult Mouse DRG

VI. TRPM8 (ALSO KNOWN AS CMR1)

Two groups have independently cloned the TRPM8 from rat TG and mouse DRG cDNAs (McKemy et al., 2002; Peier et al., 2002a). This channel is activated by cold temperatures with a threshold of \sim 23–26°C and by menthol. Northern blot analysis detected TRPM8 mRNA in the TG and DRG but not in the spinal cord, brain, skin, heart, muscle, snout, or digit. In situ hybridizsation histochemistry demonstrated the expression of this mRNA in a limited population of small TG and DRG neurons in the mouse and rat (McKemy et al., 2002; Peier et al., 2002a). In TrkA mutant mice, the expression of TRPM8 was completely abolished in the newborn DRG, suggesting that TRPM8 expression was NGF dependent, at least in early developmental periods (Peier et al., 2002a). As was expected from the size distribution, TRPM8 was expressed in unmyelinated C-fiber or lightly myelinated A δ fiber neurons that are not marked by immunohistochemistry for 150 kDa neurofilament (NF150) in the adult mouse DRG (Table III). However, none of the major subclasses of nociceptor neurons, TRPV1-, CGRP-positive, or IB4labeled neurons, express TRPM8 mRNA (Table III) (Peier et al., 2002a).

VII. TRPA1 (RENAMED FROM ANKTM1)

This distant family member of the TRP channels has been cloned from mouse TG cDNA (Story *et al.*, 2003). TRPA1 is activated by cold temperatures with a lower threshold (\sim 17°C) than that of the TRPM8 (\sim 23–26°C)


FIGURE 2 Expression of TRPM8 and TRPA1 mRNAs in rat DRG neurons. Dark-field photomicrographs of the sections hybridized with radiolabeled riboprobes for the TRPM8 and TRPA1 and visualized by autoradiography (Kobayashi *et al.*, 2005a).

and insensitive to menthol. Northern blot analysis detected TRPA1 mRNA in the DRG but not in the heart, brain, spleen, lung, liver, muscle, kidney, testis, spinal cord, whisker, or paw of the mouse and rat, suggesting that primary sensory neurons specifically express this channel just like the TRPM8. *In situ* hybridization histochemistry and immunostaining analysis demonstrated that the TRPA1 was expressed by few DRG neurons and that all of these neurons were not immunostained for NF150, similar to TRPM8. However, the distribution of the TRPA1 among other subpopulations of DRG neurons is quite different from that of the TRPM8. Almost all TRPA1-expressing neurons coexpress CGRP and TRPV1, and there is no overlap between the TRPM8-positive and TRPA1-positive subpopulations (Table III) (Story *et al.*, 2003). The coexpression of the TRPV1 and TRPA1 may be one of the molecular mechanisms responsible for why noxious cold can paradoxically be perceived as burning pain (Fig. 2).

VIII. CONCLUSIONS

There have been many reports about the expression and distribution of the TRP families in sensory neurons. However, for example, the reported percentages of the TRPV1 have been quite variable (Table I). These differences were largely due to differences in the methods as well as differences in the criteria used to differentiate positive and negative labeling. In addition, there seems to be considerable differences among species (Table I). The authors and readers must recognize these limitations of histological studies, taking care in interpreting and assigning significance to findings from such studies.

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