THE VANILLOID RECEPTOR: A Molecular Gateway to the Pain Pathway

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Abstract: The detection of painful stimuli occurs primarily at the peripheral terminals of specialized sensory neurons called nociceptors. These small-diameter neurons transduce signals of a chemical, mechanical, or thermal nature into action potentials and transmit this information to the central nervous system, ultimately eliciting a perception of pain or discomfort. Little is known about the proteins that detect noxious stimuli, especially those of a physical nature. Here we review recent advances in the molecular characterization of the capsaicin (vanilloid) receptor, an excitatory ion channel expressed by nociceptors, which contributes to the detection and integration of pain-producing chemical and thermal stimuli. The analysis of vanilloid receptor gene knockout mice confirms the involvement of this channel in pain sensation, as well as in hypersensitivity to noxious stimuli following tissue injury. At the same time, these studies demonstrate the existence of redundant mechanisms for the sensation of heat-evoked pain.

INTRODUCTION

A great deal is now known about the molecular basis of sensory perception, especially in the visual, olfactory, and gustatory systems for which cell surface proteins that detect changes in our physical or chemical environment have been identified. These molecules endow sensory neurons with specialized receptive properties, thereby determining how we perceive the world around us. Pain is also a sensory modality in which specialized primary afferent neurons, called nociceptors, detect noxious stimuli. Nociceptors are remarkable and unusual sensory cells because they respond to a broad range of physical (e.g., heat, cold, and pressure) and chemical (e.g., acid, irritants, and inflammatory mediators) stimuli, but do so only at stimulus intensities capable of causing tissue damage. However, in contrast to other sensory systems, little is known about the molecules that account for the...
unique properties of the nociceptor. Thus, a fundamental goal in pain biology is to provide a molecular understanding of how physical and chemical stimuli are detected by the nociceptor, how intensity thresholds are specified, and how these thresholds are reset in the setting of tissue injury or disease.

Our understanding of somatosensory mechanisms that detect changes in pressure, touch, or temperature is limited by the fact that receptors for these physical stimuli have not been clearly identified. Why has progress in this area lagged behind that for other sensory systems? One factor may relate to the intrinsic difficulty of carrying out biochemical studies in a system in which sensory nerve fibers and their receptors are diffusely spread throughout the body. This is in sharp contrast to the visual system, for example, in which the primary signal transduction apparatus and associated proteins are highly concentrated in one easily obtainable tissue. In the case of olfaction and taste, the sensory apparatus is also localized to a single organ, but biochemical studies are not feasible owing to the relatively small number of sensory neurons in these tissues. Nevertheless, electrophysiological and pharmacological studies have shown that signal transduction in these systems occurs through a well-characterized paradigm involving the activation of G-protein-coupled receptors, opening the way to molecular studies through homology-based screening strategies (Adler et al 2000, Buck & Axel 1991, Jones & Reed 1989, McLaughlin et al 1992). In the case of the “pain pathway,” significantly less information is available regarding the types of molecules or signal transduction mechanisms involved in the detection of noxious stimuli, particularly those of a physical nature, limiting the utility of database searches and polymerase chain reaction-based screening strategies.

Despite these limitations, pharmacological methods offer alternative strategies for initiating molecular studies, especially when a drug or agent that has robust and selective effects on the cellular or physiological system of interest is identified. Natural products from microbes, sea creatures, and plants have a spectacular track record in this regard because evolution works to maximize their capacity for seizing control of endogenous signaling pathways. Understanding how such compounds elicit their effects can yield tremendous insight into the molecular mechanisms underlying complex physiological processes. In fact, the pain pathway has provided some of the best examples of this approach, as illustrated by morphine and aspirin, two natural plant products whose analgesic actions led to the discovery of opiate receptors and cyclooxygenases, respectively (Farah & Rosenberg 1980, Snyder 1977). Similar goals have propelled research on capsaicin and related vanilloid compounds, fueling the search for a bona fide receptor and the endogenous stimuli that these compounds mimic.

HISTORICAL PERSPECTIVE

“Hot” peppers have been cultivated in South America for over 7000 years and in the rest of the world since the 16th century. Today, nearly one-fourth of the world’s
population consumes hot peppers or related foods on a daily basis (Szallasi & Blumberg 1999). Why do these peppers “burn” when we bite into them? The answer comes from the combined efforts of chemists and physiologists, who have worked on this problem for over 100 years. In the mid-19th century, Thresh isolated the principal pungent component of peppers of the genus *Capsicum* and named it capsaicin (Thresh 1846). Several decades later, Hogyes proposed that *Capsicum* extracts act selectively on sensory neurons to promote a sensation of pain and trigger heat loss through sweating (Hogyes 1878). In 1919, Nelson reported the structure of capsaicin as being an acylamide derivative of homovanillic acid, 8-methyl-N-vanillyl-6-noneamide (Nelson 1919; Figure 1). Little more was learned about the actions of capsaicin until Jancso demonstrated in the 1950s and 1960s that this compound not only activates sensory neurons but also renders animals resistant to painful stimuli (Jancso et al 1967). Since this discovery, capsaicin sensitivity has proven to be an extremely useful functional marker for a subset of neurons that are specialized to detect unpleasant or painful (noxious) stimuli. Studies of capsaicin action have provided insights into the activation of primary afferent nociceptors and have revealed the ability of some nociceptors to act in an efferent capacity by stimulating inflammation, smooth muscle contraction, or secretion in target tissues. Moreover, an appreciation of the mechanisms by which capsaicin desensitizes neurons has provided a rational basis for the use of capsaicin and related compounds in the treatment of painful disorders ranging from diabetic neuropathy to arthritis.

A number of authoritative and scholarly reviews have been written on the subjects of capsaicin pharmacology and capsaicin-sensitive neurons (Bevan & Szolcsanyi 1990, Holzer 1991, Szallasi & Blumberg 1999, Szolcsanyi et al 1994, Wood 1993). In the present review, we provide only a brief description of primary afferent neurons of the pain pathway and highlight some of the major pharmacological and physiological findings of the past 30 years that relate to capsaicin action, including evidence for the existence of specific “vanilloid” receptors. We then describe the complementary DNA (cDNA) cloning and functional characterization of a vanilloid receptor (VR1) and a homologous molecule, vanilloid receptor-like protein 1 (VRL-1). Finally, we outline the phenotypic properties of mice lacking VR1 and discuss implications for the role of VR1 in nociception.

### PRIMARY AFFERENT NEURONS OF THE PAIN PATHWAY

The cell bodies of nociceptors, like those of most other primary afferent neurons, reside in one of three locations: (a) dorsal root ganglia, which innervate the trunk, limbs, and viscera and project centrally to the spinal cord dorsal horn; (b) trigeminal ganglia, which innervate the head, oral cavity, and neck and project centrally to the brain stem trigeminal nucleus; and (c) nodose ganglia, whose peripheral terminals innervate visceral tissues and whose central terminals project to the floor of the fourth ventricle [for general reviews of nociceptor anatomy and physiology, see Fields (1987), Millan (1999), Raja et al (1999), and Snider & MacMahon (1998)].
Figure 1  Structural comparison of several molecules capable of activating or inhibiting vanilloid receptors.
Nociceptors can be found among two categories of primary afferent neurons, C fibers and Aδ fibers. C fiber nociceptors have small-diameter, unmyelinated, slowly conducting axons and small (<30 µm)-diameter cell bodies. Aδ fiber nociceptors, in contrast, have medium-diameter, lightly myelinated peripheral axons whose conduction velocities are intermediate between those of C fibers and the rapidly conducting, large-diameter Aβ fibers. Despite considerable overlap, Aδ fiber cell bodies are, on average, larger than those of C fibers (Harper & Lawson 1985). These two neuronal populations can also be distinguished by the ability of A fibers to bind monoclonal antibodies directed against neurofilament proteins (Lawson & Waddell 1985). C and Aδ nociceptors can be further subclassified on functional and anatomical grounds. Members of one group of C fibers, known as polymodal nociceptors, respond to all three pain-producing modalities (i.e. mechanical, chemical, and thermal), while others respond only to subsets of these modalities (Bessou & Perl 1969, Kumazawa & Mizumura 1977, Lang et al 1990, Lynn & Carpenter 1982). Anatomically, most C fibers fall into one of two categories (Snider & McMahon 1998). One population contains pro-inflammatory peptides such as substance P and calcitonin gene-related peptide and is regulated by nerve growth factor. The other population is nonpeptidergic but can be identified histologically by the presence of specific enzymes (e.g. thiamine monophosphate or fluoride-resistant acid phosphatase) or binding sites for the isolectin B4 (IB4). While the latter neurons are nerve growth factor-dependent during embryogenesis, their neurotrophin dependence switches during early postnatal life such that they instead require glial cell line-derived neurotrophic factor. Peptidergic and nonpeptidergic C fiber nociceptors also exhibit distinct projection patterns to the spinal cord dorsal horn, with nonpeptidergic fibers terminating at a slightly deeper layer than peptidergic fibers. It does not appear that these two anatomical populations differ from one another with regard to the stimulus modalities to which they respond. Rather, they appear to contribute differentially to the enhanced pain responsiveness that follows nerve injury as opposed to target tissue injury.

Aδ nociceptors can be functionally subdivided into two categories (Dubner et al 1977, Leem et al 1993, Meyer & Campbell 1981, Treede et al 1995). Type I Aδ nociceptors can be activated by intense mechanical stimuli or by noxious heat at temperatures higher than 52°C. Type II Aδ nociceptors are also sensitive to both mechanical and heat stimuli but exhibit a lower temperature threshold of 43°C, similar to that of nociceptive C fibers. Many Aδ nociceptors can also be directly activated or sensitized by irritant chemical stimuli such as protons, prostaglandins, or bradykinin (Lang et al 1990, Martin et al 1987, Steen et al 1992).

Capsaicin-Sensitive Primary Afferent Neurons

Capsaicin sensitivity is considered to be a principal pharmacological trait of a major subpopulation of nociceptive sensory neurons. Most capsaicin-sensitive nociceptors are C fibers (Jancso et al 1977, Szolcsanyi 1977), but another,
less numerous population consists of Aδ fibers (Lawson & Nickels 1980, Nagy et al 1983). It will be of no surprise to anyone who has handled chili peppers that the skin (Foster & Ramage 1981, Kenins 1982, Szolcsanyi 1977), cornea (Belmonte et al 1991, Szolcsanyi & Janes-Gabor 1975), and mucous membranes of the mouth (Szolcsanyi 1977, Szolcsanyi & Jancso-Gabor 1975) are rich in capsaicin-sensitive neurons. In addition, capsaicin-sensitive afferent fibers innervate the muscles (Kaufman et al 1982), joints (He et al 1988), and a host of visceral organs in the cardiovascular, respiratory, and genitourinary systems (Coleridge & Coleridge 1977, 1984; Maggi et al 1986; Szolcsanyi 1993). Visceral capsaicin-sensitive afferent neurons are involved in both reflex autonomic responses to visceral stimuli (e.g. changes in blood flow, heart rate, or respiratory rate) and the conscious perception of visceral discomfort (Ness & Gebhart 1990). Some neurons in the preoptic hypothalamus have also been reported to exhibit capsaicin sensitivity (Hori 1984, Jancso-Gabor et al 1970). It has been proposed that these cells represent “warm” receptors involved in the regulation of core body temperature.

CAPSAICIN EFFECTS ON SENSORY NEURONS

Capsaicin-Evoked Excitation

Early in vivo and in vitro physiological recordings revealed that capsaicin depolarizes sensory neurons by promoting the influx of sodium and calcium ions (Baccaglini & Hogan 1983, Heyman & Rang 1985, Marsh et al 1987, Taylor et al 1984, Williams & Zieglgansberger 1982). Whole-cell voltage clamp experiments on cultured sensory neurons (Baccaglini & Hogan 1983, Bevan & Forbes 1988, Bevan & Docherty 1993, Liu & Simon 1994) have revealed that capsaicin stimulates a cationic current response that exhibits a relative preference for divalent cations and little distinction among monovalent ions. Robust permeability to cations has also been demonstrated biochemically by measuring 45Ca2+ influx and [3H]guanidinium efflux (Wood et al 1988) and microscopically by visualizing Co2+ or Ca2+ influx with histochemical stains (Winter 1987) and calcium-sensitive fluorescent dyes (Bleakman et al 1990). Electrophysiological studies of membrane patches excised from sensory neurons have further demonstrated that capsaicin triggers the membrane-delimited opening of discrete ion channels (Bevan & Docherty 1993, Forbes & Bevan 1988, Oh et al 1996). Like whole-cell currents, these single-channel responses exhibit cation selectivity and an outwardly rectifying current-voltage relationship such that at a given absolute transmembrane potential, outward conductance is greater than inward conductance. As discussed below, substantial outward rectification is also characteristic of responses mediated by the cloned capsaicin receptor (Caterina et al 1997). This property may serve to enhance calcium influx during the depolarizing phase of the action potential spike, thereby altering action potential kinetics, calcium-dependent regulation of ion channel activity, or the release of neurotransmitters and neuromodulators from the nociceptor [for further discussion see Clapham (1997), Gunthorpe et al (2000)].
Efferent Functions of Capsaicin-Sensitive Neurons

Peripheral activation of capsaicin-sensitive afferent neurons triggers the vesicular release of glutamate and neuromodulatory peptides from their central terminals in the spinal cord dorsal horn, thereby eliciting an acute pain response. A peculiar feature of capsaicin-sensitive neurons, however, is that stimulation by capsaicin or other noxious stimuli can also trigger the release of neuropeptides from their peripheral terminals [for reviews, see Holzer (1991, 1993), Maggi (1993), and Maggi & Meli (1988)]. Chief among these neuropeptides are substance P (which triggers plasma extravasation) and calcitonin gene-related peptide (which triggers vasodilatation). The peripheral release of neuropeptides can be triggered by several routes: (a) direct activation of a peripheral capsaicin-sensitive terminal, (b) activation of a collateral terminal of the same nociceptor (accounting for the flare response commonly observed some distance from the site of tissue injury), and (c) retrograde activation of a capsaicin-sensitive afferent neuron by an antidromic electrical stimulus. While activation by the second and third routes can be blocked by local anesthetics that target voltage-gated sodium channels, peptide release occurring after the direct activation of a terminal is insensitive to these agents. Likewise, the direct stimulation of neurogenic inflammation by capsaicin does not depend on the activity of voltage-gated calcium channels, suggesting that the local calcium influx produced by vanilloid receptor activation is a sufficient stimulus for this process. What is the physiological importance of this efferent function? One obvious role is the recruitment of serum factors and inflammatory cells to promote healing at the site of injury and to ward off infection. The neurogenic release of peptides and other vesicular contents can also trigger noninflammatory responses such as secretion and smooth muscle contraction. It has therefore been suggested that tonic baseline efferent activity may serve roles unrelated to injury, such as the trophic maintenance of target tissue or the regulation of visceral tone and blood flow. However, under pathological circumstances, excessive neurogenic peptide release by capsaicin-sensitive afferent neurons is thought to contribute to the maladaptive inflammation associated with such conditions as asthma, inflammatory bowel disease, interstitial cystitis, and arthritis (Campbell 1993, Lundberg 1993). Thus, drugs that antagonize capsaicin receptor-evoked responses might be useful in the treatment of these disorders.

Capsaicin-Evoked Desensitization

Exposure to capsaicin leads initially to nociceptor firing and a period of enhanced sensitivity to painful thermal and mechanical stimuli. This phase is typically followed by a refractory period during which the individual is relatively resistant to capsaicin and certain other painful stimuli (Jancso 1992, Jancso et al 1967). Depending on the capsaicin dose, duration of treatment, route of administration, and subject age and species, this refractory state may last anywhere from hours to the lifetime of the subject. Functional changes are often accompanied
by morphological changes that range from mild swelling of axon terminals and mitochondria to complete degeneration of the neuron. In the most extreme situation, neonatal rats or mice treated systemically with 50 mg of capsaicin/kg of body weight exhibit a selective degeneration of C fiber (and some A fiber) axons and an irreversible loss of >80% of small-diameter sensory neuron cell bodies (Jancso 1984, Jancso et al 1977, Lawson & Nickels 1980, Nagy et al 1983, Scadding 1980). As adults, these animals are unresponsive to noxious chemical stimuli such as capsaicin, mustard oil, and xylene and exhibit no neurogenic inflammation in response to these compounds or to antidromic electrical stimulation of cutaneous nerves. Many investigators have reported that rats or mice treated neonatally with capsaicin also exhibit reduced responsiveness to noxious thermal and/or mechanical stimuli (Cervero & McRitchie 1981, Doucette et al 1987, Hayes et al 1981, Holzer et al 1979, Jancso 1984). However, these latter effects vary widely in the literature, possibly owing to the use of different assays of thermal and mechanical nociception and to different extents of C fiber degeneration.

Humans, too, exhibit a sequence of sensory changes with local administration of capsaicin. Intradermal or topical application of this agent results in an initial burning sensation and hyperalgesia to mechanical and thermal stimuli. This is followed by a period of decreased sensitivity to painful chemical, mechanical, or thermal stimuli, as well as mild thermal stimuli (Simone et al 1987, 1998; Simone & Ochoa 1991). Histological analysis of the injection site reveals a reduction in the number of epidermal and subepidermal nerve fibers (Simone et al 1998). These effects underlie the clinical use of cutaneous capsaicin for treatment of burning pain associated with diabetic neuropathy or human immunodeficiency virus-related neuropathy (Robbins 2000), as well as the intravesical infusion of vanilloids for the treatment of hyperactive bladder conditions associated with spinal cord injury (Chancellor & de Groat 1999).

Capsaicin-evoked desensitization and degeneration of sensory neurons can also be observed in vitro (Dray et al 1990a, Marsh et al 1987, Williams & Zieglgansberger 1982, Wood et al 1988, Yeats et al 1992). Prolonged exposure of explanted or cultured, dissociated sensory neurons to capsaicin produces an electrophysiological response that reaches a peak and then subsides despite the continued presence of agonist. Likewise, repeated application of capsaicin at short interstimulus intervals produces a series of responses that decrease in magnitude, especially between the first and second applications. In culture, the removal of extracellular calcium from the bath solution greatly reduces the extent of both electrophysiological desensitization and neuronal degeneration. Chelation of intracellular calcium with 1,2-bis(2-Aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (BAPTA) or addition of antagonists of the calcium-dependent phosphatase calcineurin also inhibits capsaicin-evoked desensitization (Cholewinski et al 1993, Docherty et al 1996, Koplas et al 1997).

One possible explanation for these results is that capsaicin-gated ion channels are more active when phosphorylated and calcium-dependent dephosphorylation therefore results in decreased channel activity. In addition, calcium-dependent
processes almost certainly contribute to capsaicin-evoked desensitization and neuronal degeneration in vivo. Indeed, neurodegeneration in capsaicin-treated animals is often accompanied by the accumulation of calcium in mitochondria (Jancso et al. 1978, 1984). Still, other processes, such as the depletion of substance P from neuronal vesicles (Yaksh et al. 1979) and nonspecific blockade of voltage-gated channels (Docherty et al. 1991, Petersen et al. 1987), are likely to contribute to capsaicin-evoked functional desensitization of nociceptors both in vitro and in vivo.

SPECIFIC VANILLOID RECEPTORS

The exquisite selectivity of capsaicin suggested early on that certain sensory neurons express a specific receptor for this compound. Initial support for the existence of a specific capsaicin “site” was provided by the observation that capsaicin analogues exhibit structure-activity relationships (Szolcsanyi & Jancso-Gabor 1975, 1976; Walpole & Wrigglesworth 1993). Further support came from the study of the phorbol ester derivative resiniferatoxin (Figure 1). This compound, produced by the cactuslike plant *Euphorbia resinifera*, is a far more potent irritant than other phorbol esters, an observation which led Blumberg & colleagues to recognize that resiniferatoxin and capsaicin are structurally related by virtue of a common vanillyl moiety (deVries & Blumberg 1989, Szallasi & Blumberg 1989). Experimentally, they and others (Winter et al. 1990) found that resiniferatoxin exhibits excitatory and desensitizing properties similar to those exhibited by capsaicin but at 1000-fold-lower doses. This relatively high specific activity makes [3H]resiniferatoxin a viable radioligand for identifying saturable, capsaicin-displaceable binding sites on membranes derived from dorsal root ganglia (Szallasi & Blumberg 1990, Winter et al. 1993). Taken together, these observations support the notion that capsaicin and resiniferatoxin activate sensory neurons by binding to specific vanilloid receptors. The identification of [3H]resiniferatoxin binding sites on the peripheral terminals, cell bodies, and central terminals of sensory neurons (Acs et al. 1994, Szallasi et al. 1995, Winter et al. 1993) corroborated functional studies showing vanilloid sensitivity at all of these subcellular locations (Heyman & Rang 1985, Such & Jancso 1986, Yaksh et al. 1979). Final pharmacological support for the existence of a bona fide vanilloid receptor came from the development of capsazepine (Figure 1), a competitive antagonist of both vanilloid binding and vanilloid-evoked responses (Bevan et al. 1992).

Molecular Cloning of the Vanilloid Receptor VR1

Whole-cell and excised-membrane patch clamp recordings from sensory neurons suggested that vanilloid receptors either have intrinsic ion channel activity or promote the opening of cationic channels through a membrane-delimited signaling mechanism. However, these studies provided no specific information about the
molecular structure of vanilloid receptors or associated ion channels. A screening strategy based on function was therefore chosen as a relatively unbiased and potentially straightforward approach for obtaining a functional cDNA encoding this receptor (Caterina et al 1997). Two assumptions were essential for the success of this screen: (a) activation of vanilloid receptors in nonneuronal cells would lead to an increase in cytoplasmic calcium levels, and (b) the product of a single cDNA would be sufficient to confer functional sensitivity to capsaicin in such a heterologous cellular environment. With these caveats in mind, cDNA pools consisting of several thousand independent clones from a rodent dorsal root ganglion library were transiently expressed in human embryonic kidney-derived HEK293 cells. Transfected cells were loaded with a calcium-sensitive dye (Fura-2) and microscopically examined for capsaicin-evoked increases in intracellular free calcium by the use of a standard ratiometric imaging system. Once identified, a positive cDNA pool was iteratively subdivided and rescreened by this facile and sensitive assay until a single clone (VR1) that rendered HEK293 cells responsive to capsaicin or resiniferatoxin was obtained.

For VR1 to fulfill the identity of a bona fide vanilloid receptor, several functional and anatomical criteria had to be met. The cloning strategy ensured that the most basic element—sensitivity to vanilloid agonists—was satisfied at the outset. This was validated by direct electrophysiological measurements in which VR1-expressing HEK293 cells or *Xenopus* oocytes showed large membrane current responses to bath-applied capsaicin or resiniferatoxin with electrophysiological properties resembling those of native vanilloid receptors, including a nonselective cation flux with relatively high membrane permeability to calcium, an outwardly rectifying current-voltage relationship at both the whole-cell and single-channel levels, and calcium-dependent desensitization (Caterina et al 1997). Moreover, these responses were attenuated by capsazepine or by ruthenium red, a presumptive pore blocker that noncompetitively antagonizes capsaicin-evoked responses in neurons (Dray et al 1990b). Relative potencies and Hill coefficients for these pharmacological agents at the cloned receptor are similar to values reported for native vanilloid receptors on rat sensory neurons (Caterina et al 1997, Tominaga et al 1998, Jerman et al 2000). The same is true for apparent binding affinities as determined by displacement of [3H]resiniferatoxin from VR1-transfected HEK293 or Chinese hamster ovary cell membranes (Szallasi et al 1999). Indeed, even vanilloid-evoked excitotoxicity can be conferred by VR1, as evidenced by the finding that HEK293 cells expressing this protein are rapidly and efficiently killed after exposure to capsaicin (Caterina et al 1997).

As mentioned above, vanilloid sensitivity is a hallmark of many small-to medium-diameter neurons in dorsal root, trigeminal, and nodose sensory ganglia, and exposure to capsaicin in vitro or in vivo can lead to excitotoxic death of these cells. Thus, one would expect VR1-specific nucleic acid or antibody probes to label a significant fraction of these cells. Double-labeling studies have shown that VR1 is indeed expressed by the majority of unmyelinated peptidergic and nonpeptidergic
neurons within these ganglia and can therefore account for the vanilloid sensitivity of these cells (Tominaga et al 1998, Guo et al 1999, Michael & Priestly 1999). It is interesting that VR1 immunoreactivity is not localized to any one region of the primary afferent neuron but is found throughout the cell (including the soma and both the peripheral and central terminals), consistent with functional studies mentioned above.

Within the spinal cord, VR1 staining is prominent in superficial regions of the dorsal horn (laminae I and II), consistent with known central projection patterns of most C fibers and binding profiles observed with [3H]resiniferatoxin. At the same time, VR1 antibodies have revealed previously undetected heterogeneity among sensory neurons, particularly within the IB4-positive class. These cells send their axons to the inner zone of lamina II (Iii), but VR1 antibodies label only the subset of IB4-positive fibers that terminate in the medial part of Iii, not those that terminate more laterally (Tominaga et al 1998). Because medial and lateral regions of the lumbar dorsal horn receive input from distal and proximal regions of the hindlimb, respectively, these observations suggest that neurochemically and functionally heterogeneous subpopulations of primary afferent neurons innervate topographically distinct regions of the body. Additional signs of anatomical segregation have been reported by Elde & colleagues, who found that VR1-positive fibers in the spinal cord or skin showed surprisingly little costaining with substance P and calcitonin gene-related peptide antibodies (Guo et al 1999). It is currently unclear how this observation can be reconciled with the extensive colocalization seen in cell bodies or with the ability of capsaicin to promote neuropeptide release from central and peripheral C fiber terminals. On a similar note, vanilloid receptors have recently been reported to exist throughout the brain, as determined by polymerase chain reaction, in situ hybridization, and immunohistochemical staining methods (Mezey et al 2000, Sasamura et al 1998, Schumacher et al 2000). However, this result is controversial because [3H] resiniferatoxin binding sites are not found on central nervous system neurons (at least not to an extent that would match proposed levels of VR1 expression) and there are no reports of vanilloid-evoked electrophysiological responses in the brain. It is conceivable that closely related subtypes were detected in this analysis, a possibility that can now be addressed by using VR1-deficient mice for similar expression studies.

**VR1 Defines a New Subfamily of TRP-Like Ion Channels**

The predicted amino acid sequence of VR1 reveals that it is a member of a growing family of ion channels first identified in the fly visual pathway. In 1969, Benzer & colleague described a mutation in *Drosophila melanogaster* that renders fruit flies blind. Electoretinograms showed that whereas normal fly eyes produced a sustained depolarizing response to a flash of light, these mutants exhibited only transient responses to light, earning them the name *trp for transient receptor potential* (Hotta & Benzer 1969). Montell & Rubin later cloned the *TRP* locus and found that
it encodes a protein with six putative transmembrane domains plus an additional short hydrophobic region connecting transmembrane segments 5 and 6 (Montell & Rubin 1989). This polytopic arrangement resembles the proposed core structure of voltage-gated potassium channels of the \textit{Shaker} family or cyclic-nucleotide-gated ion channels from olfactory neurons and vertebrate photoreceptor cells. TRP channels also contain multiple ankyrin repeats within their N-terminal cytoplasmic region, a signature feature that is shared by VR1 and many (but not all) members of this growing family of cation channels [for a recent review of TRP channel structure and function, see Harteneck et al (2000)]. Three homologous TRP channel subunits have been found in the \textit{Drosophila} eye, where they are thought to form several different homotetrameric and heterotetrameric channels. TRP homologues have now been identified in many organisms, ranging from worms to humans, and in cell types ranging from sensory neurons to lung cells. On the basis of its predicted primary amino acid sequence, VR1 defines its own subfamily of mammalian TRP-like channels, which is now known to include at least two additional members (see below). The closest invertebrate homologue appears to be the product of the \textit{osm-9} gene from the worm \textit{Caenorhabditis elegans} (Colbert et al 1997). Behavioral phenotypes associated with mutations in the \textit{osm-9} gene have implicated this channel in signaling pathways that detect odorants, mechanical stimuli, or changes in osmotic strength.

How are TRP channels activated? In the insect eye, photostimulation of rhodopsin results in the G-protein-dependent activation of phospholipase C. This enzyme, which cleaves phosphatidylinositol bisphosphate into inositol trisphosphate (IP$_3$) and diacylglycerol, somehow triggers the gating of TRP channels, allowing sodium and calcium to enter and depolarize the photoreceptor cell (Ranganathan et al 1995). Heterologous-expression studies have demonstrated that vertebrate members of the TRP channel family can also be gated by occupancy of G-protein-coupled receptors (Zhu et al 1996) or neurotrophin receptors (Li et al 1999) that stimulate phospholipase C. The precise mechanism(s) by which these channels are gated, however, has been the subject of much debate. Some have suggested that certain TRP channels act as “store-operated” calcium channels that are gated by the phospholipase C-mediated depletion of IP$_3$-sensitive intracellular calcium stores (Berridge 1995, Friel 1996). It has been further suggested that this gating mechanism involves a direct or indirect conformational coupling between TRP channels and the IP$_3$ receptor, analogous to that between dihydropyridine receptors and ryanodine receptors in muscle (Berridge 1995, Kiselyov et al 1998). Other studies, however, suggest a role for the diacylglycerol limb of the phospholipase C signaling pathway in TRP channel activation. For example, genetic studies in fruit flies have shown that TRP channels can be activated in the absence of IP$_3$ receptors (Acharya et al 1997, Scott & Zucker 1998). Moreover, several groups have now shown that fruit fly and mammalian TRP channels can be directly activated in excised membrane patches by diacylglycerol or its derivatives, such as arachidonic or linoleic acid (Chyb et al 1999, Hofmann et al 1999). These findings suggest that polyunsaturated fatty acids or other structurally related lipids may serve as
endogenous ligands for TRP channels in the fruit fly eye or elsewhere, an issue that we discuss in greater detail below.

As mentioned above, VR1 is the founding member of a growing subfamily of mammalian TRP channels. With the aid of database searches, we identified a cDNA sharing ~50% sequence identity with VR1 (Caterina et al 1999). HEK293 cells and *Xenopus* oocytes expressing this clone are insensitive to capsaicin, protons, and moderate heat but do show nonselective cationic currents when challenged with a high-temperature stimulus exceeding a threshold of ~52°C. This novel channel was dubbed vanilloid receptor-like protein 1 (VRL-1) to reflect its structural similarity to VR1 but its insensitivity to capsaicin. Within sensory ganglia, VRL-1 is expressed primarily by medium- to large-diameter neurons, making it a candidate transducer of high-threshold heat responses by these cells, which probably constitute the lightly myelinated Aδ nociceptors of the type I class. VRL-1 transcripts are also found in a variety of other tissues, including spinal cord, spleen, brain, and lung, suggesting that this channel is activated by physiological stimuli other than heat. In fact, a mouse orthologue of VRL-1 [termed growth-factor-regulated channel (GRC)] was subsequently identified in a polymerase chain reaction-based screen for novel growth factor-regulated channels (Kanzaki et al 1999). Chinese hamster ovary cells transfected with this clone show increased cell surface expression of GRC and enhanced permeability to calcium after exposure to insulin-like growth factor. Thus, the VRL-1/GRC channel may be regulated by growth factors through mechanisms involving increased translocation to the plasma membrane, second-messenger-mediated changes in channel activity, or both. A similar concept may apply to another, more distantly related member of this TRP subfamily, referred to as an epithelial calcium channel (ECaC), which shares <30% identity with VR1 (Hoenderop et al 1999). ECaC was cloned from a rabbit kidney library by screening for cDNAs that enhanced calcium uptake in *Xenopus* oocytes. The pharmacological and physiological properties of ECaC suggest that it constitutes a vitamin D₃-regulated apical calcium channel activity characterized in kidney epithelium.

VRL-1 and ECaC show structural similarity to VR1, but they are clearly encoded by separate genes. In addition to these homologues, a number of cDNAs that share large regions of nucleotide identity with VR1, and probably represent alternatively spliced transcripts derived from the VR1 gene, have been described. One of these variants is called SIC, a putative stretch-inhibitable channel that is missing a significant portion of the VR1 N terminus (the coding region begins at amino acid position 308 of VR1) and shows divergence within the C-terminal cytoplasmic domain (Suzuki et al 1999). Another N-terminally truncated variant, VR.5′sv, is predicted to begin at the same position as SIC but is otherwise identical to VR1 (Schumacher et al 2000). Heterologous cells transfected with VR.5′sv cDNA do not respond to capsaicin, protons, or heat. Thus, it is not yet clear whether these putative splice variants yield functional channels or complex with VR1 or related molecules to produce channels having novel pharmacological or electrophysiological properties in vitro or in vivo.
Figure 2  Cationic flux through VR1 can be regulated by the convergent actions of multiple pain-producing stimuli. Membrane-penetrant second messengers, such as lipid metabolites, may modulate nociceptor activity in an autocrine fashion (as diagrammed here), or in a paracrine manner if produced by neighboring neural or nonneural cell types. Abbreviations: AA, arachidonic acid; G, heterotrimeric G protein; GPCR, G protein-coupled receptor; PL, phospholipase. Capsaicin and noxious heat are represented by pepper and flame, respectively.

VR1 IS A POLYMODAL DETECTOR OF NOXIOUS PHYSICAL AND CHEMICAL STIMULI

A major impetus to identifying the capsaicin receptor was to determine whether known pain-producing stimuli might exert their effects by acting at this same site. With a functional vanilloid receptor cDNA in hand, this fascinating question could finally be addressed by challenging VR1-expressing cells with a variety of chemical and physical stimuli capable of activating primary afferent neurons in vitro or in vivo. As summarized below, these studies have produced a number of interesting candidates for physiologically relevant vanilloid receptor agonists (Figure 2).

Heat

Many proteins, including receptors and channels, show alterations in their structure or activity as a function of temperature, but VR1 has thermal response characteristics that make it especially interesting in the context of nociception. Most notably, VR1 is gated by heat, but only when ambient temperatures exceed ~43°C (Tominaga et al 1998), a threshold matching that of heat-evoked pain responses
in humans and animals or heat-evoked electrophysiological responses in primary afferent nerve fibers or cultured sensory neurons (Cesare & McNaughton 1996, LaMotte & Campbell 1978, Raja et al 1999). Evidence for a direct relationship between VR1 expression and heat sensitivity is supported by several observations (Tominaga et al 1998), the most basic of which is that sensitivity to capsaicin and heat sensitivity are significantly correlated (in both frequency and magnitude) among VR1-transfected HEK293 cells. Responses to these stimuli also show cross-desensitization, and both capsaicin- and heat-evoked currents are attenuated by the vanilloid receptor antagonists capsazepine and ruthenium red. In addition, both responses are characterized by outwardly rectifying current-voltage relations and relatively high permeability to calcium ions. Some distinctions do exist, such as quantitative differences in cation permeability ratios or requirements for extracellular calcium in desensitization, suggesting that vanilloids and heat activate VR1 through overlapping but distinct mechanisms. In VR1-transfected HEK293 cells (as in sensory neurons), capsaicin or heat evokes single-channel currents in excised membrane patches, demonstrating that channel activation occurs via a membrane-delimited mechanism that does not require the action of diffusible cytoplasmic second messengers (Tominaga et al 1998). Site-specific mutations in VR1 can significantly alter capsaicin potency or thermal activation thresholds (Jordt et al 2000), providing further evidence that VR1 itself transduces responses to these stimuli when expressed in heterologous cells.

These observations of heterologous nonneuronal systems have led us to propose that VR1 functions as a molecular transducer of noxious thermal stimuli in vivo. Consistent with this hypothesis, sensitivity to capsaicin and sensitivity to noxious heat are also well correlated among small-diameter sensory neurons in culture (Kirschstein et al 1997; Nagy & Rang 1999a,b). Moreover, VR1 and native heat-evoked currents have a number of properties in common, including similar current-voltage relationships (Cesare & McNaughton 1996, Nagy & Rang 1999b, Reichling & Levine 1997), selective permeability to cations (Cesare & McNaughton 1996, Nagy & Rang 1999b, Reichling & Levine 1997), and, in some studies, sensitivity to vanilloid receptor antagonists (Kirschstein et al 1999). At the same time, numerous discrepancies between native heat- and vanilloid-evoked responses have been reported, including differences in relative permeability to calcium and sodium ions (Cesare & McNaughton 1996, Nagy & Rang 1999b) and sensitivity to vanilloid receptor antagonists (Nagy & Rang 1999b, Reichling & Levine 1997). Moreover, Nagy & Rang have recently shown that the amplitudes of the capsaicin- and heat-evoked responses among individual sensory neurons are not tightly correlated, contrary to the scenario expected if the same channel responds to both stimuli. Most significantly, capsaicin- and heat-evoked responses showed poor cosegregation at the single-channel level in membrane patches excised from cultured rat sensory neurons (Nagy & Rang 1999b). That is, most patches were sensitive to capsaicin or heat, but only a few patches responded to both stimuli (although the frequency of dually responsive patches was significantly higher than one would predict for random inclusion of two independent channels in the same patch).
From these findings, Nagy & Rang proposed that distinct ion channels respond to capsaicin and heat. These channels could consist of entirely different molecules or different functional isoforms of VR1 generated through alternative RNA splicing, post-translational modification, or association with other cellular proteins. Unfortunately, similar patch clamp analyses have not been carried out with VR1-expressing HEK293 cells; such experiments might help to address these issues.

At least some of the apparent discrepancies listed above may be accounted for by the fact that sensory neurons express multiple forms of heat-activated channels that differ in their biophysical or pharmacological properties. Indeed, as described above a recently identified VR1 homologue (VRL-1) is insensitive to capsaicin or protons but does respond to high-threshold heat stimuli (>50°C) when expressed in nonneuronal cells (Caterina et al 1999). This threshold is similar to that reported for a subset of medium- to large-diameter sensory neurons in culture (Nagy & Rang 1999a) and for some thin myelinated (Aδ) nociceptors in vivo (Raja et al 1999, Treede et al 1995). In fact, VRL-1 expression within rat and mouse sensory ganglia is confined primarily to neurons with these same anatomical properties (Caterina et al 1999). We have therefore proposed that VRL-1 accounts for the “high-threshold” thermal sensitivity of this subset of nociceptors while VR1 detects moderate-intensity heat stimuli in small-diameter, unmyelinated (C fiber) nociceptors. However, this model is based largely on correlative evidence, owing to the paucity of selective and potent pharmacological agents with which to manipulate vanilloid receptors in vivo.

Protons

Tissue damage, such as that associated with infection, inflammation, or ischemia, produces an array of chemical mediators that activate or sensitize nociceptor terminals to elicit pain and promote tenderness at the site of injury (Handwerker & Reeh 1991, Levine & Taiwo 1994). Protons constitute one important component of this pro-algesic response, reducing the extracellular pH to levels below the physiological norm of ~7.6. Extracellular protons elicit both transient and sustained excitatory responses in cultured sensory neurons, the latter of which is believed to account for persistent pain associated with local tissue acidosis (Bevan & Geppetti 1994). Protons are capable of modulating the activity of a number of receptors and ion channels expressed by primary afferent nociceptors, including acid-sensitive channels of the degenerin family (Chen et al 1998; Lingueglia et al 1997; Waldmann et al 1997a,b), ATP-gated channels (Li et al 1997, Stoop et al 1997), and vanilloid receptors (Caterina et al 1997, Jordt et al 2000, Kress et al 1996, Martenson et al 1994, Petersen & LaMotte 1993, Tominaga et al 1998). Which, if any, of these entities contributes to acid-evoked pain is presently unclear, but electrophysiological and genetic studies of native and cloned vanilloid receptors suggest that they play a significant role in mediating sustained proton responses in vivo.

Because protons have been proposed to act as modulators of native vanilloid receptors, there is significant interest in understanding the relationship between
sensitivity to capsaicin and proton sensitivity at the cellular and molecular levels. We have shown, using both VR1-expressing mammalian cells and *Xenopus* oocytes, that moderately acidic bath conditions augment capsaicin-evoked responses by increasing agonist potency (50% effective concentration = 90 nM at pH 7.4 versus 36 nM at pH 6.4) without altering efficacy (Caterina et al. 1997, Jordt et al. 2000, Tominaga et al. 1998). Importantly, extracellular protons also potentiate heat-activated currents (Tominaga et al. 1998). Temperature response curves generated in VR1-expressing oocytes or HEK293 cells show that a reduction in extracellular pH produces markedly larger responses at temperatures that are noxious to mammals (>43°C). Moreover, a reduction in pH dramatically lowers the threshold for channel activation, such that at pH 6.3, substantial currents can be seen at temperatures as low as 35°C, conditions under which the channel is normally closed (at pH 7.6). This augmentation of VR1 thermal responsiveness by protons closely resembles the increase in nociceptor thermal sensitivity associated with inflammation (Handwerker & Reeh 1991). In both cases, there is a significant decrease in the threshold for heat-evoked responses and an increase in response magnitudes at temperatures above the initial pain threshold. Importantly, VR1 shows especially dynamic modulation of heat-evoked currents between pH 8 and 6 (Jordt et al. 2000), a sensitivity range that matches the extent of local acidosis attained during most forms of tissue injury. Below pH 6, sustained membrane currents can be observed in VR1-expressing HEK293 cells at room temperature (22°C), with a half-maximal effective pH of 5.4 (Tominaga et al. 1998). Whether these proton-evoked responses result simply from a decrease in the channel’s thermal response threshold or involve additional steps is not entirely clear, but recent structure-function studies suggest that proton-evoked channel activation and proton-mediated potentiation can be functionally uncoupled (Jordt et al. 2000; see below).

How do protons modulate VR1 activity? Electrophysiological studies of native and cloned receptors suggest that extracellular protons act primarily to increase the probability of channel opening (Tominaga et al. 1998, Baumann and Martenson 2000) rather than by increasing unitary conductance or interacting directly with a vanilloid binding site (which may be intracellular (Jung et al. 1999)). In fact, acidic bath solutions evoke ionic currents when applied to outside-out, but not inside-out, membrane patches excised from VR1-expressing HEK293 cells, suggesting that protons interact with an extracellular site(s) on the channel complex (Tominaga et al. 1998). Candidate sites for such interactions include several acidic amino acids located within putative extracellular loops of VR1. Site-directed mutational analysis has pinpointed two glutamate residues of particular interest, one at position 600 and another at position 648 (Jordt et al. 2000). The first of these residues is located between the putative fifth transmembrane domain and pore loop segment. Introduction of neutral or positive residues at this site increases the responses of VR1-expressing cells to capsaicin or heat. For example, E600Q mutant channels show a >10-fold increase in sensitivity to capsaicin (no change in efficacy) and E600K mutants show a dramatic decrease in thermal activation threshold
(30–32°C). Conversely, introduction of a more acidic residue at this position (E600D) decreases sensitivity to these stimuli. Thus, E600 appears to play a critical role in determining the pH sensitivity range of channel activation by noxious stimuli. Interestingly, mutants bearing nontitratable amino acids at position 600 (e.g. E600Q) can still be activated by low-pH solutions, suggesting that proton-evoked channel activation and proton-mediated potentiation involve titration of different sites. Further support for this idea comes from mutational analysis of another putative extracellular glutamate residue. Oocytes expressing E648A mutant channels are essentially insensitive to activation by low-pH (4.0) solutions but retain sensitivity to capsaicin and heat and show normal proton-mediated potentiation of these stimuli. The selective nature of this phenotype also suggests that protons, vanilloids, and heat promote channel opening through distinct pathways having one or more stimulus-specific steps. These site-directed mutagenesis studies provide additional evidence that protons interact with specific amino acids on the extracellular surface of VR1 to allosterically modulate channel activity. However, they do not by themselves pinpoint exact sites of titration by extracellular protons.

Lipids

Capsaicin bears structural similarity to a number of lipid-derived second messengers (e.g. arachidonic acid), suggesting that VR1 may be activated by an endogenous ligand of this sort. Indeed, capsazepine and other competitive vanilloid antagonists were developed with the idea that they might block receptor activation under circumstances (e.g. inflammation) in which endogenous capsaicin-like agonists would be produced (Bevan et al 1992). A number of recent observations, both from within and outside the vanilloid receptor field, provide compelling arguments for the existence of endogenous capsaicin-like ligands that may activate VR1 or alter its sensitivity to other stimuli.

One line of evidence comes from the analysis of phototransduction in the fruit fly eye. As mentioned above, Drosophila TRP can be activated in vitro by polyunsaturated fatty acids, such as arachidonic or linoleic acid (Chyb et al 1999). Moreover, heterologously expressed mammalian TRPC3 and TRPC6 channels can be activated by diacylglycerol (Hofmann et al 1999), further implicating lipids as potential in vivo regulators of TRP channel function. Another line of evidence comes from structural and functional connections between cannabinoid and vanilloid receptor pharmacology. For example, synthetic vanilloid receptor ligands, such as olvanil or other long-chain N-acyl-vanillyl amides, bear structural similarity to the endogenous cannabinoid receptor agonist anandamide (arachidonylethanolamide; Figure 1). Olvanil also resembles AM404 (Figure 1), a synthetic anandamide transport inhibitor, and both block reuptake of anandamide into cells (Melck et al 1999, Beltramo and Piomelli 1999). It therefore follows that some cannabinoid receptor ligands might interact with vanilloid receptors. Indeed, this turns out to be the case for both anandamide and AM404 (Zygmunt et al 1999, 2000a). These
compounds are significantly less potent than capsaicin, and they elicit responses with somewhat slower kinetics, but both evoke outwardly rectifying, nonselective cationic currents in HEK293 cells or *Xenopus* oocytes expressing VR1. These responses appear to be specific because they are inhibited by capsazepine, but not by cannabinoid receptor antagonists, and a number of other synthetic or endogenous cannabinoid receptor agonists have little or no effect on VR1 function.

In each case in which bioactive lipids have been shown to modulate TRP-like channels in vitro, the estimated 50% effective concentration falls in the range of $10^{-7}$ to $10^{-5}$ M (the 50% effective concentration for anandamide activation of VR1 is $\sim 5 \mu$M). This raises questions as to whether such concentrations are attained under normal or pathophysiological conditions. Of course, in vitro electrophysiological estimates of agonist potency are determined by perfusing aqueous suspensions of these hydrophobic agents over a cell surface or membrane bilayer, but how much actually reaches the appropriate site on the channel protein is difficult, if not impossible, to estimate. Polyunsaturated fatty acids or structurally related metabolites are produced in vivo through enzymatic cleavage of membrane lipids, and their access to receptors within the same or adjacent cells may occur with significantly higher efficiency, particularly if much of the action is confined to the hydrophobic environment of the bilayer. Moreover, under inflammatory conditions, macrophages and endothelial cells may release substantial amounts of anandamide and other lipid messengers into a confined intercellular space, such that the local concentrations of these agents may approach the micromolar range.

Anandamide produces analgesia with potency in the nanomolar range and inhibits release of calcitonin gene-related peptide in the skin through its actions at cannabinoid receptors. These findings have led some to argue that anandamide exerts its actions on sensory neurons via cannabinoid receptors alone, without the involvement of vanilloid receptors (Szolcsanyi 2000). However, modulation of sensory neuron function by these receptor systems need not be viewed as a mutually exclusive possibility since anandamide may interact with VR1 to produce physiological effects that are distinct from those mediated via cannabinoid receptors [see Smart & Jerman (2000) and Zygmunt et al (2000b) for more-detailed discussions]. The principal action of anandamide on vanilloid receptors may be to potentiate responses to other stimuli, most notably heat or protons. In fact, under conditions of elevated temperatures or decreased pH, the potency of anandamide as a VR1 agonist may be significantly increased. Thus pro-algesic agents such as ATP, bradykinin, serotonin, leukotrienes, and prostanoids may sensitize the primary afferent neuron, in part, by stimulating the production of lipid-derived second messengers that sensitize VR1 and consequently enhance nociceptor excitability. Additionally, anandamide produced by nonneuronal cells may synergize with these agents by modulating the activity of VR1 channels on neighboring sensory nerve terminals. Finally, anandamide may be just one of several as-yet-unidentified lipids capable of activating VR1. VR1-expressing cell lines provide the necessary tools to characterize novel, and possibly more potent, lipid agonists by means of calcium imaging, electrophysiological, or radioligand binding.
assays. In fact, a high-throughput screen of >1000 bioactive substances reidentified anandamide as an activator of VR1 (Smart et al 2000), and a directed analysis of arachidonic acid metabolites identified lipooxygenase products [e.g. 12- or 15-(S)-Hydroperoxyeicosa-5Z, 8Z, 11Z, 13E-tetraenoic acid] as agonists of this channel (Hwang et al 2000). Maximal efficacies for these compounds differ by as much as fivefold, and all of the substances exhibit potencies in the 1–10 µM range at room temperature.

Where on the VR1 protein do vanilloids and cannabinoids bind, and how do they promote channel opening? Sites of interaction have not yet been mapped, and little is known about the mechanism(s) of channel gating. Capsaicin can activate VR1 when it is applied to either side of an excised membrane patch (Caterina et al 1997), consistent with the idea that vanilloids can permeate or cross the lipid bilayer to mediate their effects. Recent electrophysiological studies using hydrophilic capsaicin derivatives suggest that vanilloids interact with an intracellular site on VR1 (Jung et al 1999), but specific ligand binding domains have not been mapped. Although capsazepine blocks the actions of both capsaicin and anandamide, it is not clear whether these compounds compete for binding to the same site. While capsaicin potency is clearly enhanced under moderately acidic (pH 6.4) conditions (Caterina et al 1997, Tominaga et al 1998), this appears not to be the case for anandamide (Smart et al 2000), suggesting that these agonists interact with VR1 in nonidentical ways. Further molecular and biochemical studies will be required to resolve these interesting questions.

GENETIC ANALYSIS OF CAPSAICIN RECEPTOR FUNCTION IN VIVO: VR1 Knockout in Mice

The studies described above present a strong circumstantial case for the involvement of VR1 not only in the actions of vanilloid compounds but also in sensory responses to noxious heat, acid stimuli, and perhaps endogenous lipids. One approach to addressing this issue directly has been to generate “knockout” mice in which the VR1 gene is disrupted (Caterina et al 2000, Davis et al 2000). These mice are viable and fertile and exhibit a normal appearance as well as normal gross behavior. Despite the absence of VR1, sensory ganglion development (as assessed by the presence of several histological markers) is apparently unaltered in these animals. Functionally, however, disruption of the VR1 gene produces an array of specific defects related to nociception, as described below.

Deficits in Cellular Physiology and Acute Nociception

Neurons derived from the dorsal root ganglia of VR1-null mice exhibit no vanilloid-evoked electrophysiological responses either in culture or in sensory nerve fibers innervating an excised patch of skin (the excised-skin nerve preparation). In the intact mouse, these deficits are manifest as drastic reductions in paw licking and
neurogenic inflammation evoked by intraplantar injection of either capsaicin or resiniferatoxin. Sensory neurons of the trigeminal system also depend on VR1 for vanilloid responsiveness: whereas wild-type mice avoid the consumption of capsaicin-containing water, littermates lacking VR1 exhibit no such aversion. Moreover, VR1-null mice fail to exhibit the profound hypothermia observed in wild-type mice after subcutaneous injection of capsaicin. Taken together, these data demonstrate that VR1 is essential for transducing the nociceptive, inflammatory, and hypothermic effects of vanilloid compounds.

The involvement of VR1 in proton-evoked nociceptive responses is supported by data from multiple in vitro assays. Whereas 30% of cultured wild-type dorsal root ganglion neurons exhibit large, sustained current responses following exposure to pH 5 medium, <7% of neurons from VR1-null mice exhibit such responses. These results are mirrored in the skin nerve preparation, in which the absence of VR1 results in an ~90% reduction in the proportion of acid-sensitive C fiber nociceptors. Most likely, the residual proton-evoked responses in VR1-null-mouse C fibers are mediated by members of the acid-sensing ion channel (ASIC) family (Chen et al 1998; Langueglia et al 1997; Waldmann et al 1997a, b). These proteins are part of a superfamily of channels that also includes the amiloride-sensitive epithelial sodium channels, peptide-gated ion channels from snails, and the so-called degenerins, putative mechanosensory channels first identified in C. elegans. At least five different ASIC subtypes have been identified, and many are expressed in sensory afferent neurons. Indeed, the expression of ASIC3 is largely restricted to these cells.

To what extent do VR1 and ASICs, respectively, contribute to proton-evoked pain or other in vivo responses associated with tissue acidification? Ischemic, inflammatory, or infectious events commonly reduce the local tissue pH to <7, with reductions sometimes to <6. VR1 can be strongly activated by a pH of 5.9 at 22°C or by a pH of 6.4 at 37°C, making it well poised to detect these tissue insults. Some ASIC family members, either alone or in combination, can also respond to protons in this concentration range, although many are activated only at lower pH values. Retrograde labeling of cardiac afferent neurons, followed by electrophysiological recording of labeled cells, has revealed that many afferent fibers innervating myocardium are capsaicin-insensitive neurons whose acid-evoked current responses most closely resemble those of heterologously expressed ASIC3 (Benson et al 1999). Thus, in this physiological setting, ASICs may play a predominant proton-sensing role. Still, the profoundly reduced prevalence of proton-evoked responses in neurons derived from VR1-null mice strongly suggests that these animals will exhibit deficits in acid-evoked sensory excitation in other visceral, muscular, or cutaneous locations.

Thermal nociception in VR1-null mice has also been examined, using a collection of in vitro and in vivo assays. Voltage-clamped, cultured sensory neurons derived from wild-type rats or mice exhibit one of three response patterns during exposure to a brief heat ramp to 60°C (Caterina et al 2000, Nagy & Rang 1999a). Approximately half of these neurons do not respond to the heat stimulus.
Another 40% exhibit large, inward currents once the temperature exceeds \( \sim 45^\circ \text{C} \). These latter cells are capsaicin sensitive, consistent with the notion that responses to both stimuli are mediated by VR1. The final 10% of neurons respond to heat only at temperatures \( >52^\circ \text{C} \). This high threshold, together with the capsaicin insensitivity of these cells, suggests that VR1-1 may account for this final class of responses. Consistent with this (perhaps oversimplified) interpretation, sensory neurons derived from \( VR1 \)-null mice exhibit a normal prevalence of the high-threshold heat-evoked current responses but none of the moderate-threshold responses.

Deficient heat-evoked responses among the sensory neurons of \( VR1 \)-null mice are also observed in single-unit recordings from the skin nerve preparation. Here, however, the picture is more complex. Among C fiber nociceptors, those of \( VR1 \)-null mice exhibit a reduction in the proportion of heat-responsive units, dropping from the wild-type incidence of 13 in 24 to 4 in 24. The remaining heat-responsive C fibers derived from \( VR1 \)-null mice exhibit a normal threshold but a reduced firing rate at higher noxious temperatures. In contrast to these deficits, mechanical sensitivity in this preparation is unaffected by the absence of VR1 (Caterina et al 2000).

\( VR1 \)-null mice also exhibit a selective reduction in thermal nociceptive input to the spinal cord dorsal horn (evoked by heating of the hindpaw), without concomitant decreases in mechanically evoked input. Curiously, thermonociceptive input to the more deeply situated wide-dynamic-range neurons of the spinal cord dorsal horn appears to be more drastically affected by the absence of VR1 than is input to projection neurons in the more superficial layers of the dorsal horn (Caterina et al 2000). This finding may reflect distinct integration by these neurons of information from VR1-positive versus VR1-negative primary afferent neurons.

In one of the knockout studies (Caterina et al 2000), it was found that \( VR1 \)-null mice exhibited selective deficits in several behavioral assays of acute heat-evoked nociception, including the tail immersion, hot plate, and radiant paw heating assays. In each case, heat-evoked withdrawal at relatively low noxious temperatures was comparable with that of wild-type littermates, while at higher temperatures examined, \( VR1 \)-null mice exhibited a longer withdrawal latency, consistent with impaired thermal nociception. Indeed, the absence of statistically significant differences in heat-evoked responses between genotypes in the study by Davis & colleagues (Davis et al 2000) most likely stems from the relatively low noxious temperatures used in that study. An analysis of this knockout strain at higher temperatures and in a homogeneous background might therefore resolve this apparent discrepancy. The selective impairment of heat-evoked nociception at high temperatures in \( VR1 \)-null mice (and preservation at lower noxious temperatures) is consistent with the relatively greater deficit in C fiber heat coding at higher temperatures but is apparently at odds with the relatively moderate in vitro threshold for VR1 activation and the absence of moderate-threshold heat-evoked responses in cultured sensory neurons derived from \( VR1 \)-null mice. Several conclusions arise from these findings. First, not all functional properties of nociceptors are faithfully recapitulated once they are dissociated and placed in culture. Second, it appears
that one cannot necessarily predict the quantitative range of a sensory deficit in a knockout-mouse model from the response properties exhibited by a single receptor molecule. At present, the significance of this observation is unclear, particularly since we do not yet know the identities or properties of all receptor molecules involved in thermosensation. These findings do, however, underscore the complexity of behavioral “readouts” of nociception, as well as the value of approaching the analysis of knockout mice from multiple physiological levels.

In any case, the findings of both VR1 knockout studies make it clear that there exist VR1-independent mechanisms for the detection of noxious heat. VRL-1 is one candidate mediator of these responses (Caterina et al 1999). The profile of heat-evoked currents observed in cultured sensory neurons derived from VR1-null mice is consistent with this explanation. However, the temperature threshold exhibited by residual heat-sensitive C fibers in VR1-null mice is significantly below that reported for recombinant VRL-1. In addition, immunofluorescence studies of rat dorsal root ganglia have suggested that VRL-1 is not expressed at detectable levels in C fibers. Thus, species differences in the pattern of VRL-1 expression and an altered VRL-1 activation threshold must be invoked to account completely for the residual heat-evoked responses observed in the VR1-null mice, unless other molecules act as receptors for noxious heat. The generation and analysis of VR1/VRL-1 doubly null mice, as well as the identification and characterization of additional vanilloid receptor homologues, might clarify this situation.

**Deficits in Tissue Injury-Induced Thermal Hyperalgesia**

As described above, electrophysiological studies of the cloned vanilloid receptor have revealed that VR1 is responsive to multiple stimuli, including heat, protons, and lipid metabolites. Injury brings on many changes that affect the activity of the nociceptor, including local tissue acidosis and the production of pro-algesic agents, such as bradykinin, ATP, monoamines, and arachidonic acid metabolites. The net result is one in which response thresholds to noxious stimuli are decreased, thereby contributing to the development of thermal and mechanical hypersensitivity. The capacity of VR1 to detect and integrate information from diverse physical and chemical inputs makes this channel potentially well suited for assessing the physiological environment of the primary afferent nerve terminal and for altering nociceptor excitability in the setting of tissue injury. We have therefore hypothesized that VR1 contributes to peripheral mechanisms underlying thermal hypersensitization.

This prediction is clearly borne out by the analyses of VR1-deficient mice. In these studies, tissue injury was elicited by treatment of the hindpaw with an inflammatory agent or nerve injury was produced by partial ligation of the sciatic nerve. Wild-type mice exhibit increased sensitivity to both thermal and mechanical stimuli after such treatments. In contrast, VR1-null mice show hypersensitivity to mechanical stimuli, as well as normal thermal hypersensitivity following nerve injury, but they do not show increased sensitivity to thermal stimuli following tissue injury. Thus, VR1 appears to be essential for the development of thermal
 hypersensitivity associated with tissue inflammation, but not that associated with nerve injury. This selective phenotype represents what may be the most significant practical outcome of the genetic studies because it highlights physiological settings in which vanilloid receptor antagonists have the potential to serve as effective analgesic agents.

Clearly, inflammation-induced thermal hypersensitivity may result from the convergent actions on VR1 of heat, low pH, and other inflammatory mediators. However, other mechanisms, such as up-regulation of VR1 expression or sensitization of VR1 by post-translational modification may also come into play. Moreover some of the effects of inflammation might also be manifest downstream of VR1, at the level of general nociceptor excitability. Further mechanistic studies will be required to dissect these possibilities.

FUTURE PROSPECTS

The study of vanilloid receptors has provided valuable insights into the molecular mechanisms by which nociceptors evaluate their physical and chemical environments. Still, it is clear from the data presented above that much more remains to be learned about these processes. For instance, what are the molecules that account for the residual heat-evoked nociception observed in VR1-null mice? What are the relative contributions of different heat sensors to the subjective experiences of warmth and heat-evoked pain? Do protons or endogenous lipids regulate vanilloid receptor activity in vivo? How are heat, protons, and lipids mechanistically integrated by VR1? What molecules account for the perception of mechanically evoked pain? Finally, how are the levels and/or activities of these molecules changed in pathological pain states? The continued integration of molecular, anatomical, behavioral, and physiological approaches in the study of pain should allow these questions to be answered.

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