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The Cloned Capsaicin Receptor Integrates Multiple Pain-Producing Stimuli

Makoto Tominaga,* Michael J. Caterina,* Annika B. Malmberg,† Tobias A. Rosen,* Heather Gilbert,† Kate Skinner,† Brigitte E. Raumann,* Allan I. Basbaum,† and David Julius*‡

*Department of Cellular and Molecular Pharmacology
†Departments of Anatomy and Physiology
and W. M. Keck Foundation Center for Integrative Neuroscience
University of California
San Francisco, California 94143

Summary

Capsaicin, the main pungent ingredient in "hot" chili peppers, elicits burning pain by activating specific (vanilloid) receptors on sensory nerve endings. The cloned vanilloid receptor (VR1) is a cation channel that is also activated by noxious heat. Here, analysis of heat-evoked single channel currents in excised membrane patches suggests that heat gates VR1 directly. We also show that protons decrease the temperature threshold for VR1 activation such that even moderately acidic conditions (pH $\leq$ 5.9) activate VR1 at room temperature. VR1 can therefore be viewed as a molecular integrator of chemical and physical stimuli that elicit pain. Immunocytochemical analysis indicates that the receptor is located in a neurochemically heterogeneous population of small diameter primary afferent fibers. A role for VR1 in injury-induced hypersensitivity at the level of the sensory neuron is presented.

Introduction

In mammals, the initial detection of noxious chemical, mechanical, or thermal stimuli, a process referred to as nociception, occurs predominantly at the peripheral terminals of specialized, small diameter primary afferent neurons, called polymodal nociceptors (Fields, 1987). These afferents transmit the information into the CNS, ultimately evoking a perception of pain or discomfort and initiating appropriate protective reflexes. The protective function of this system is predicated on its ability to detect diverse physical and chemical stimuli, distinguish between noxious and innocuous events by setting specific response thresholds for a given stimulus, and reset these thresholds to sensitize the system and guard against further injury. A fundamental goal in pain biology is to understand how disparate physical and chemical stimuli are qualitatively and quantitatively sensed at the nociceptor terminal under normal and pathophysiological conditions. Do noxious thermal, mechanical, and chemical stimuli excite nociceptors through shared or distinct signal transduction pathways? How does a response to one type of stimulus alter sensitivity to others? To address these questions, it is necessary to identify the molecular entities at the nociceptor terminal that detect noxious signals and transduce this information into membrane depolarization events.

Our approach to identifying such molecules has been to elucidate the mechanism whereby plant-derived agents, such as capsaicin, resiniferatoxin, and other vanilloid-containing compounds, elicit a sensation of burning pain. Vanilloid compounds selectively depolarize nociceptors (Bevan and Szolcsanyi, 1990; Szolcsanyi, 1993; Szallasi, 1994), presumably by mimicking the actions of a physiological stimulus or endogenous ligand that activates the "nociceptive" pathway. We have recently determined the molecular basis underlying this phenomenon by characterizing a functional cDNA that encodes a vanilloid receptor (VR1) in rat sensory ganglia (Caterina et al., 1997). VR1 is a vanilloid-gated, nonselective cation channel that resembles members of the transient receptor potential (TRP) channel family, first identified as components of the *Drosophila* phototransduction pathway (Montell and Rubin, 1989). The restriction of VR1 expression to small diameter neurons within sensory ganglia is sufficient to account for the highly selective nature of vanilloid compounds as excitatory agents for nociceptors. Remarkably, *Xenopus* oocytes or transfected mammalian cells expressing VR1 exhibit robust membrane currents not only in response to capsaicin but also to increases in ambient temperature capable of producing pain in humans (i.e., noxious heat). Because vanilloid- and heat-evoked currents are cation selective, have similar outwardly rectifying current–voltage relations, and are blocked by the noncompetitive capsaicin receptor antagonist ruthenium red, we have postulated that VR1 mediates responses to both stimuli in vitro and in vivo (Caterina et al., 1997). However, the relationship between heat- and vanilloid-evoked currents at the whole-cell and single channel levels remains unclear. Moreover, it has yet to be established whether heat activates VR1 directly or through other thermally sensitive molecules. Finally, a number of studies conducted in cultured sensory neurons have provided evidence for heterogeneity among heat-evoked currents (Cesare and McNaughton, 1996; Reichling and Levine, 1997), and the relationship of VR1 to these activities remains to be resolved.

In addition to heat and vanilloid compounds, protons also influence vanilloid receptors and nociceptive pathways. High proton concentrations (pH $<$ 6) are generated during various forms of tissue injury, including infection, inflammation, and ischemia (Jacobus et al., 1977; Stevens et al., 1991; Steen et al., 1992; Bevan and Geppetti, 1994). Such acidification can elicit pain or sensitize the affected area (Steen et al., 1992; Steen and Reeh, 1993; Bevan and Geppetti, 1994). In the latter case, a stimulus above the normal pain threshold can evoke an exaggerated pain response, a condition referred to as hyperalgesia (Fields, 1987; Dubner and Basbaum, 1994; Meyer et al., 1994). Protons are likely to produce or exacerbate pain via their interaction with several receptors and channels on nociceptive sensory neurons, including acid-sensitive ion channels of the

‡To whom correspondence should be addressed.

degenerin family (Waldmann et al., 1997a, 1997b), ATP-gated ion channels (Stoop et al., 1997), and vanilloid receptors (Petersen and LaMotte, 1993; Kress et al., 1996; Caterina et al., 1997). In cultured sensory neurons, protons potentiate capsaicin-evoked responses (Petersen and LaMotte, 1993; Kress et al., 1996). At higher concentrations, protons by themselves elicit membrane currents (Bevan and Yeats, 1991; Kress et al., 1996), a component of which has been postulated to result from their direct or indirect activation of vanilloid receptors (Bevan and Yeats, 1991; Bevan and Geppetti, 1994).

With the cloned capsaicin receptor in hand, we can now directly address the mechanisms of action of heat, protons, and vanilloid compounds on the receptor and explore the relationships among these stimuli at the cellular and molecular levels. In the present study, we provide evidence, in the form of single channel recordings from excised membrane patches and simultaneous current and temperature recordings in oocytes, that heat activates VR1 directly and dynamically without the involvement of cytoplasmic components. We also show that protons dramatically potentiate the effects of heat and capsaicin on VR1 activity by decreasing the threshold for channel activation by either agonist. In fact, at physiologically attainable proton concentrations ($pH < 5.9$), the channel opens at room temperature. In light of these observations, we present a model wherein the vanilloid receptor functions as a polymodal signal detector whose activity reflects the combined status of multiple physiological stimuli. Finally, using antisera directed against the cloned receptor, we provide evidence that VR1 is expressed both centrally and peripherally within somatic and visceral unmyelinated primary afferent nociceptors. We also show that VR1 is not uniformly expressed in peptide- (substance P) and non-peptide- (lectin IB4) containing dorsal root ganglion (DRG) neurons, which suggests that there is a heretofore undetected physiological heterogeneity among polymodal nociceptors. The anatomical distribution and functional properties that we describe are consistent with VR1 contributing to both thermosensation and chemosensation in vivo. Moreover, the ability of VR1 to detect and integrate information from physical and chemical inputs is a property expected of a signal transduction molecule that assesses the physiological environment of the sensory nerve terminal and alters neuronal responsiveness in the context of tissue injury.

Results

Functional Comparison of Capsaicin- and Heat-Activated Responses

We conducted a series of electrophysiological and pharmacological analyses to better understand the functional relationship between capsaicin- and heat-evoked VR1 currents. In transfected human embryonic kidney–derived HEK 293 cells expressing VR1, we found that the magnitudes of capsaicin-evoked (1 μM) and heat-evoked (46°C) currents in individual cells were significantly correlated ($r = 0.82$, $p < 0.0001$, least squares regression, $n = 26$; data not shown), with heat-evoked responses being ~25% the size of those evoked by capsaicin. This correlation is in good agreement with the reported concordance between heat- and capsaicin-evoked responses in cultured sensory neurons (Kirschstein et al., 1997) and is consistent with VR1 being a direct effector for both stimuli. Second, we asked whether activation by one agonist would cross-desensitize VR1 to the other agonist. To answer this question, we initially examined responses in the absence of extracellular calcium, where repetitive capsaicin-evoked currents do not desensitize (Caterina et al., 1997) but repetitive heat-evoked currents show significant desensitization (Figure 1A). Under these conditions, we found that heat produced profound cross-desensitization to subsequent challenges with capsaicin (Figure 1A). Similarly, we found that, in the presence of extracellular calcium, capsaicin treatment desensitized VR1-expressing cells not only to subsequent challenges with capsaicin but also to heat (Figure 1A). Interestingly, desensitization to either agonist could be overcome by applying both stimuli simultaneously. The resulting responses desensitized in a calcium-dependent manner, similar to the behavior of capsaicin responses in naive cells. Third, we asked whether heat-evoked currents exhibited the distinctive cation permeability sequence ($Ca^{2+} > Mg^{2+} > Na^{+}$) that is characteristic of capsaicin-evoked currents (Bevan and Szolcsanyi, 1990; Caterina et al., 1997). Ion substitution experiments revealed that heat-evoked currents show a similar preference for divalent cations, albeit with somewhat different relative permeabilities: for heat, the ratios were $P_{Ca}/P_{Na} = 3.8 \pm 0.3$, $n = 6$, and $P_{Mg}/P_{Na} = 3.1 \pm 0.4$, $n = 6$ (data not shown); for capsaicin, they were $P_{Ca}/P_{Na} = 9.6$ and $P_{Mg}/P_{Na} = 5.0$ (Caterina et al., 1997). Fourth, we tested whether antagonists that block capsaicin-evoked responses would also block heat-evoked currents. Consistent with our previous observations in oocytes, we found that both the noncompetitive antagonist ruthenium red (10 μM) and the competitive antagonist capsazepine (10 μM) significantly blocked capsaicin-evoked responses in VR1-expressing HEK 293 cells (Figure 1B). Likewise, heat-activated currents in transfected cells were virtually abolished in the presence of these antagonists, compared with a 44% decrease (attributable to desensitization between the first and second heat challenges) in the absence of antagonist (Figures 1B and 1C). Moreover, capsazepine (10 μM) produced a significant blockade of heat-activated currents in VR1-expressing *Xenopus* oocytes that partially recovered following antagonist washout (Figure 1C). Taken together, these observations strongly support the conclusion that VR1-mediated responses to capsaicin and heat involve distinct but overlapping mechanisms.

Heat Activates VR1 by a Membrane Delimited and Graded Mechanism

If VR1 is, itself, a heat sensor, then heat-evoked responses should occur in isolated membrane patches, free of soluble cytoplasmic components. Indeed, well-resolved heat- or capsaicin-evoked currents were observed in patches excised from VR1-transfected (but not vector-transfected) HEK 293 cells in the inside-out (Figures 2A and 2B) or outside-out (data not shown)

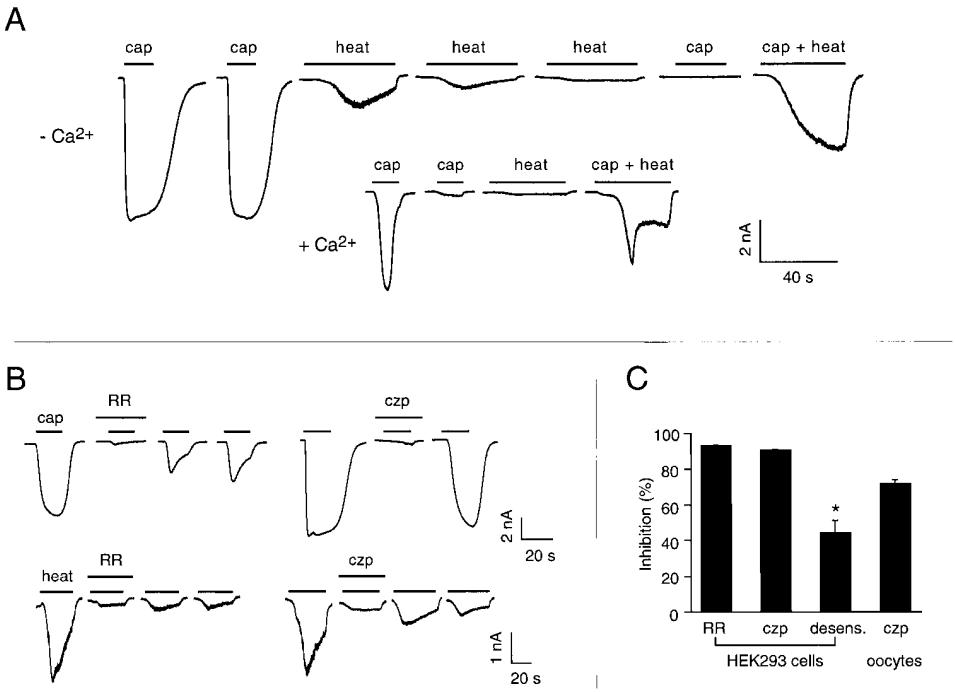

Figure 1. The Cloned Capsaicin Receptor Is an Effector for Both Vanilloid Compounds and Heat

(A) Capsaicin and heat show cross-desensitization and synergistic recovery. Whole-cell current responses were evoked by capsaicin (cap) or heat in VR1-transfected mammalian HEK 293 cells bathed in Ca^{2+}-free or Ca^{2+}-containing solutions. Desensitization produced by 1 μM capsaicin in Ca^{2+}-containing solution could not be overcome by application of 100 μM capsaicin alone (data not shown). No significant heat-evoked currents were observed in cells expressing $5HT_3$ (n = 5) or $P2X_2$ (n = 5) ligand-gated channels (data not shown).

(B and C) Vanilloid receptor antagonists block both capsaicin- and heat-evoked currents. Antagonism by capsazepine (czp, 10 μM) or ruthenium red (RR, 10 μM) was assessed following a 30 s antagonist pretreatment period for capsaicin-evoked responses and a 2 min pretreatment period for heat-evoked response. Capsaicin-activated currents in HEK 293 cells were blocked by RR (98.1% ± 1.0%, n = 7) or czp (97.3% ± 1.7%, n = 7). Heat-activated currents in HEK 293 cells were also inhibited by RR (92.2% ± 1.0%, n = 4) and czp (90.3% ± 0.5%, n = 5). Decrease of heat-activated currents in the absence of antagonist due to receptor desensitization between the first and second heat challenges (desens.) in HEK 293 cells (43.7% ± 7.3%, n = 8) was significantly smaller than the apparent inhibition in the presence of antagonist (*$p < 0.001$, unpaired t test). Heat-activated currents in oocytes were inhibited by czp (71.2% ± 2.9%, n = 11). Capsaicin concentration was 1 μM and bath temperature was elevated from ~22°C to 46°C in 25 s (monitored using an in-bath thermocouple) and from ~22°C to 44°C in HEK 293 cells and oocytes, respectively. Holding potentials (E_h) were −60 mV and −40 mV in HEK 293 cells and oocytes, respectively. The interstimulus interval was 2 min; bars indicate the duration of agonist stimuli.

configuration. These single channel responses displayed a number of properties in common: (1) both showed reversible inhibition by capsazepine (10 μM), as reflected in a significant reduction in the mean channel activity (NP_o) (Figure 2A); (2) both exhibited outwardly rectifying current–voltage relations (Figure 2B and Caterina et al., l997); and (3) single channel Na^+ conductances derived from these current–voltage relations were similar for heat- and capsaicin-evoked currents (83 and 77 pS, respectively, at positive potentials; Figure 2B and Caterina et al., 1997). These rectification and conductance properties resemble those determined for native heat-activated currents in cultured sensory neurons (Cesare and McNaughton, 1996; Reichling and Levine, 1997; H. Rang, personal communication).

If VR1 directly senses heat stimuli, then one might also expect heat-activated currents to vary directly as a function of ambient temperature. In oocytes expressing VR1, heat-evoked currents were remarkably responsive to changes in bath temperature above 40°C. Continuous increases and decreases in temperature produced coordinate changes in current, as if the channel were acting as a molecular thermometer (Figure 2C). Taken together with the single channel data, these findings indicate that any mechanism underlying VR1 activation must involve a membrane delimited signaling event in which channel responses are temporally linked to changes in thermal stimuli. In the simplest scheme, VR1 is an intrinsically heat-sensitive channel that can respond to thermal stimuli in any cellular environment.

Protons Potentiate Both Capsaicin- and Heat-Evoked Responses

The observation that a drop in bath pH (pH_o) augments responses of cultured sensory neurons to capsaicin (Petersen and LaMotte, 1993; Kress et al., 1996) has raised many questions about the pharmacological mechanism that underlie this phenomenon. Is it relevant, for instance, to the process whereby acidosis exacerbates pain? In initial experiments, we found that protons enhance the response of VR1-expressing oocytes to capsaicin (Caterina et al., 1997). The same effect is seen in VR1-transfected mammalian cells, and dose–response

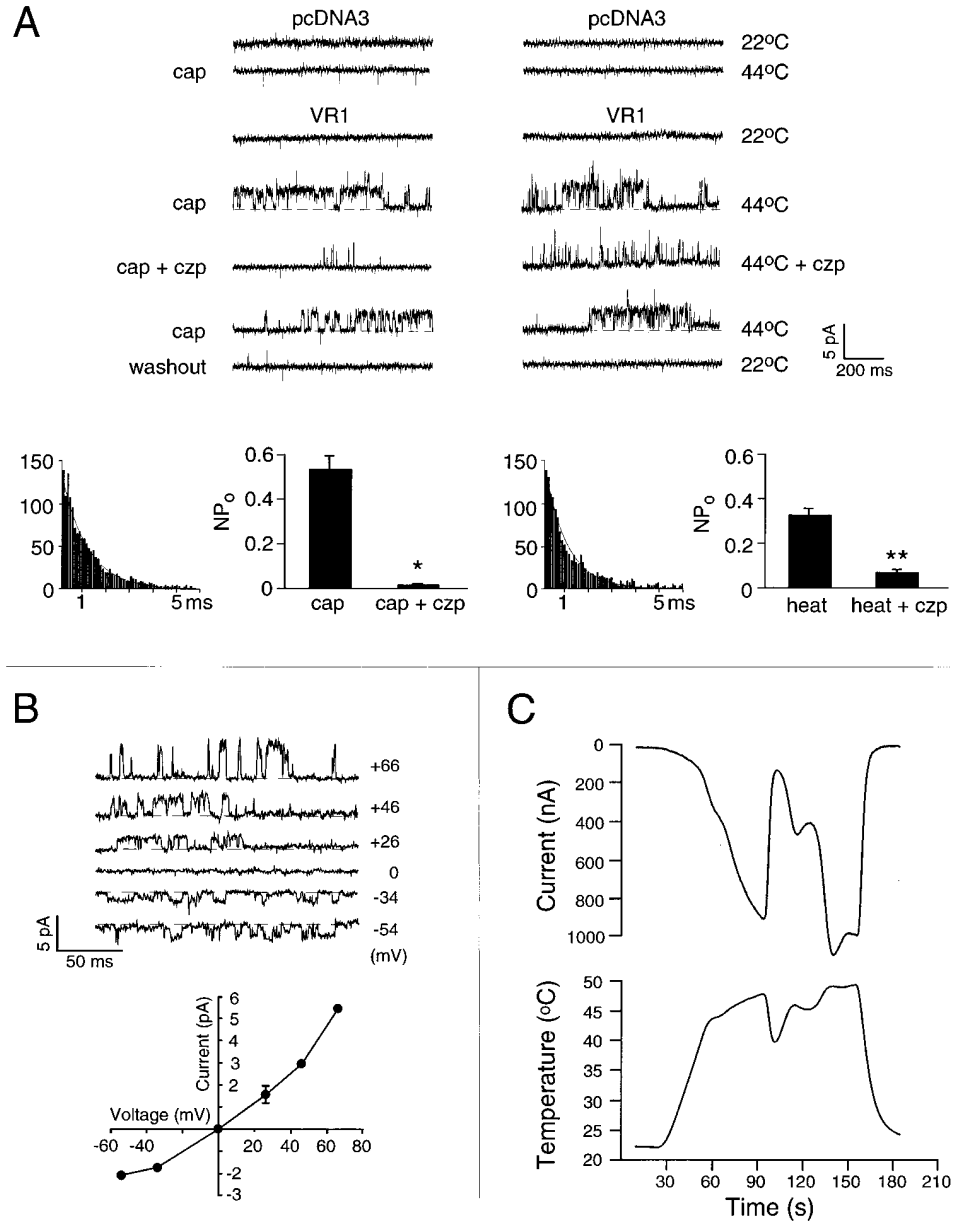

Figure 2. Heat Activates VR1 through a Membrane-Delimited and Graded Mechanism

(A) Capsaicin (cap, left, 100 nM) and heat (right, 44°C) evoke single channel openings in VR1-transfected but not in vector-transfected (pcDNA3) control cells. Single channel activity elicited by capsaicin was significantly blocked by capsazepine (czp, 10 μM, NP_o reduced by 97.7% from 0.528 ± 0.058 to 0.012 ± 0.004, n = 7, *$p < 0.0001$, paired t test) as was heat-evoked activity (NP_o reduced by 79.8% from 0.321 ± 0.029 to 0.065 ± 0.012, n = 11, **$p < 0.00001$, paired t test). Unitary amplitudes were 2.87 ± 0.09 pA for capsaicin (+40 mV, n = 10) and 2.95 ± 0.07 pA for heat (+46 mV, n = 28). Open time histograms from representative patches are shown at bottom; time bins are 0.1 ms. Time constants obtained by fitting histograms to single exponentials were not significantly different for capsaicin (0.98 ± 0.10 ms, n = 4) and heat (0.86 ± 0.04 ms, n = 5, unpaired t test). For heat-activated currents, membrane potentials were corrected for the junctional potential change measured at 44°C (6.3 ± 0.4 mV, n = 6). Broken lines indicate closed channel level.

(B) Heat-evoked single channel currents show outward rectification. Representative single channel traces of heat-activated currents (44°C) at indicated membrane potentials are shown (top) together with derived current–voltage curve of mean single channel amplitudes (± SEM) (bottom, n = 5–28 patches). Broken lines indicate closed channel level. Single channel chord conductance for Na^+ between 0 and +66 mV was 83.4 ± 2.9 pS (n = 5).

(C) Fluctuations in bath temperature above 40°C elicit dynamic and synchronized current changes in voltage-clamped VR1-expressing oocytes.

curves generated under neutral (pH 7.4) and acidic (pH 6.4) bath conditions clearly demonstrated that protons potentiate capsaicin action by increasing its potency (EC_{50} = 111 nM and 60 nM, respectively) without altering efficacy (Figure 3A). Under these moderately acidic conditions, protons serve only as modulatory agents because no current was observed in the absence of capsaicin (data not shown).

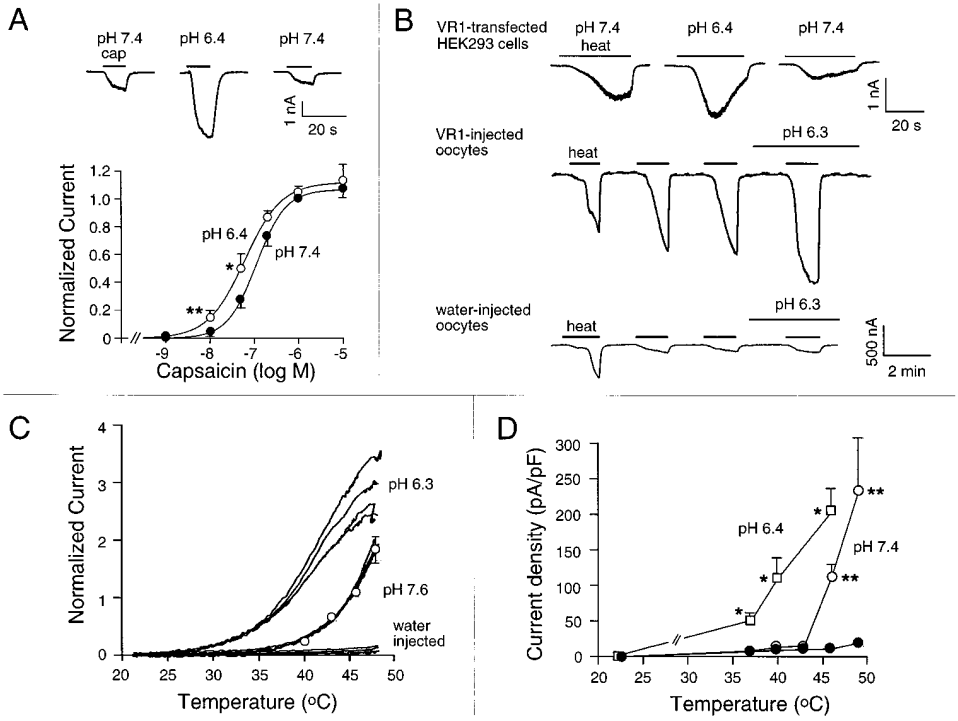

Figure 3. Protons Potentiate Capsaicin- and Heat-Activated VR1 Currents

(A) A change in extracellular pH from 7.4 to 6.4 reversibly potentiates whole-cell currents activated by capsaicin (cap, 50 nM) at room temperature. Capsaicin (bars) was applied at 2 min intervals, and cells were pretreated with pH 6.4 bath solution for 2 min. Concentration–response curves for capsaicin in bath solutions of pH 7.4 (closed circles) and pH 6.4 (open circles) exhibit a 1.9-fold difference in agonist potency. EC_{50} values were 111 nM (SD = 16 nM) and 60 nM (SD = 14 nM); Hill coefficients were 1.32 (SD = 0.21) and 1.02 (SD = 0.21), respectively. Normalized whole-cell currents (mean ± SEM, n = 14) are expressed as a fraction of the response evoked in each cell by 1 μM capsaicin at pH 7.4. Asterisk and double asterisk indicate significant differences between pH 7.4 and pH 6.4 (*$p < 0.05$ and **$p < 0.01$, unpaired t test).

(B) Protons potentiate heat-activated currents in VR1-expressing HEK 293 cells (top) and oocytes (middle) held at −60 and −40 mV, respectively. In HEK 293 cells, bath temperature was elevated to 46°C as in Figure 1. Pretreatment time with pH 6.4 bath solution was 2 min. In oocytes, bath temperature was elevated from ~22°C to 48°C over 90 s. Heat-evoked responses in oocytes, which increased in size between the first and second stimuli, stabilized by the third stimulus. Water-injected control oocytes (bottom) sometimes exhibited an initial heat-evoked current that was markedly diminished during subsequent challenges. Room temperature bath solution at pH 6.3 evoked no response in VR1- or water-injected oocytes (data not shown).

(C and D) Protons lower the threshold and increase the magnitude of heat-evoked currents in VR1-expressing cells. Temperature- response curves in oocytes (C) were derived from the increasing temperature phase of the third (pH 7.6) and fourth (pH 6.3) heat-evoked responses in cells treated as in (B). For a given VR1-expressing oocyte (n = 4), currents were normalized to responses at 45°C (pH 7.6). For water-injected control oocytes (n = 4), the third (pH 7.6) heat-evoked response was plotted as a fraction of the average 45°C, pH 7.6 stimulus-evoked current among VR1-expressing oocytes. Open circles indicate mean (± SEM) normalized current measured after 30 s of continuous exposure to fixed temperatures (n = 4). Temperature-response curves in transfected HEK 293 cells (D) were derived in bath solutions of fixed temperatures at pH 7.4 (open circles, n = 6–17) or pH 6.4 (open squares, n = 7–9). Vector-transfected cells were similarly treated at pH 7.4 (closed circles, n = 5–7). Heat (46°C) evoked no responses at low pH in vector-transfected HEK 293 cells (data not shown). Each point represents mean values (± SEM). Current was normalized to membrane capacitance. Asterisks and double asterisks indicate significant difference from VR1-expressing cells at pH 7.4 and from vector-transfected cells at pH 7.4, respectively ($p < 0.01$, unpaired t test).

Of greater physiological relevance, of course, is whether protons modulate the response of VR1 to heat. In fact, we found that a decrease in extracellular pH significantly potentiates heat-evoked responses in either VR1-expressing HEK 293 cells or *Xenopus* oocytes (Figure 3B). To determine whether this effect occurs over a range encompassing both noxious and nonnoxious temperatures, we generated continuous temperature-response curves from oocytes expressing VR1. In this system, membrane currents were recorded from voltage-clamped oocytes while the temperature of the perfusate solution was elevated from ~22°C to 48°C. Unlike mammalian cells, oocytes expressing VR1 exhibited minimal desensitization of heat-evoked responses. As a result, a thermal response curve generated during a continuous temperature ramp was superimposable with one defined by currents obtained at several steady state temperatures (Figure 3C). In other words, continuous temperature ramps can be used to generate accurate thermal response profiles of VR1 in oocytes. These temperature-response curves illustrate two interesting and significant points. First, a reduction in pH_o produces markedly larger responses at temperatures that are noxious to mammals. Second, a reduction in pH_o dramatically lowers the threshold for channel activation, such that at pH_o 6.3, substantial currents can be seen at temperatures as low as 35°C (where the channel is normally closed at pH_o 7.6). We observed a similar phenomenon

in VR1-transfected mammalian cells exposed to heat stimuli at fixed temperatures (Figure 3D). Here, a clear leftward shift of the temperature–response curve was also seen with a reduction in bath pH. Notably, the threshold for heat-evoked responses at pH_o 7.4 was ~43°C, in excellent agreement with reported thermal response thresholds for heat-activated currents in cultured rat sensory neurons (Cesare and McNaughton, 1996), subjective pain ratings in humans, and pain-associated single-fiber activity in monkeys (LaMotte and Campbell, 1978; Meyer and Campbell, 1981; Meyer et al., 1994). Moreover, the graded response of VR1 above this threshold is analogous to progressively larger physiological responses observed in these systems with further increases in temperature.

Protons Activate VR1 at Normal Physiological Temperatures

To more precisely mimic a physiological scenario in which inflammation or ischemia lowers tissue pH at body temperature, we exposed VR1-transfected cells to an abrupt decrease in pH_o (to 6.4) at a constant temperature of 37°C, which is well below the temperature threshold for activating VR1 at normal physiological pH. Under these circumstances, a distinct proton-evoked current was observed (Figure 4A), demonstrating that at the nonelevated body temperature of 37°C, protons can act as VR1 agonists. In light of this observation, we examined the effects of protons over a broader range, speculating that at sufficiently high concentrations, hydrogen ions might further shift the VR1 thermal response profile, rendering the channel open at room temperature. As predicted, sustained proton-evoked responses were reproducibly observed at pH_o 5.4 in VR1-transfected (but not vector-transfected) mammalian cells at 22°C. Responses to capsaicin and protons shared several characteristics, including calcium-dependent desensitization profiles, reversible inhibition by capsazepine, and outwardly rectifying current–voltage relations (Figure 4B and Caterina et al., 1997). A dose–response curve generated at 22°C exhibited a threshold pH of $\leq$5.9 with a half-maximal effective pH of 5.4 (Figure 4C). We estimate that saturating proton concentrations produce maximal responses that are 20%–30% of the magnitude of responses obtained with a saturating dose of capsaicin (1.0 μM), which suggests that protons behave as partial agonists of VR1 with respect to capsaicin. We previously reported that <10% of VR1-expressing oocytes showed currents upon exposure to pH 5.5 bath solution (Caterina et al., 1997). Here, we found that when pH_o was reduced to 4.0, responses were uniformly observed (data not shown). The reason for the reduced sensitivity of VR1 to protons in this system is not clear but could reflect differential membrane lipid composition, posttranslational modifications, or interactions of VR1 with other cellular components in mammalian versus amphibian cells.

In membrane patches excised from VR1-transfected (but not vector-transfected) HEK 293 cells, protons evoked discrete single channel openings (Figure 4D). These responses exhibited a unitary amplitude (at +40 mV) similar to that observed for heat-evoked and capsaicin-evoked responses. Interestingly, acidified bath solution elicited responses only when applied to outside-out membrane patches, suggesting that protons interact with a region of the receptor that resides on the extracellular face of the plasma membrane. Candidate sites for proton interaction include several acidic residues found within putative extracellular loops of the VR1 protein. Neutralization of these residues by protonation might alter VR1 function by destabilizing hydrophilic interactions and/or stabilizing hydrophobic interactions that favor ion channel opening. Taken together, our observations demonstrate that, in mammalian cells, extracellular protons activate VR1 in the absence of cytoplasmic components and in a concentration range readily achieved during tissue injury.

Distribution of VR1 Protein

Capsaicin sensitivity is probably the best functional marker of C-fiber nociceptors (Jancso et al., 1977; Martin et al., 1987; Szolcsanyi et al., 1988), and, indeed, VR1 transcripts are found exclusively in small to medium diameter primary sensory neurons (Caterina et al., 1997). Antibodies to the VR1 protein would therefore provide an important molecular marker for these cells and facilitate the anatomical analysis of the primary afferent component of the "pain" pathway. We therefore raised a rabbit antiserum against a synthetic peptide corresponding to the predicted VR1 carboxyl terminus. Immunoblot analysis of whole-cell lysates from VR1-transfected HEK 293 cells revealed a tight doublet migrating at ~90 kDa, close to the predicted molecular mass of VR1 (Figure 5F). This doublet was not observed in lysates from vector-transfected control cells. Addition of immunizing peptide eliminated the doublet, whereas addition of an unrelated peptide had no effect, confirming the specificity of the antiserum.

Our immunocytochemical analysis focused on the distribution of VR1 in the spinal cord and caudal brainstem, where primary sensory neurons send their central projections from dorsal root and trigeminal ganglia, respectively (Marfurt, 1981; Molander and Grant, 1985). In general, the pattern of immunostaining was comparable to that observed when the capsaicin receptor was localized by binding of [^{3}H]-resiniferatoxin (Winter et al., 1993; Acs et al., 1994; Szallasi et al., 1995). Intense immunoreactivity was observed in the terminals of afferent fibers projecting to the superficial layers of the spinal cord dorsal horn (Figures 5A and 5C) and the trigeminal nucleus caudalis (Figure 5B). In the spinal cord, the densest staining was found in laminae I and II, although some labeled axons extended ventrally to the neck of the dorsal horn and around the central canal (lamina V and X, respectively). Projections to laminae V and X arose from axons that coursed along the medial edge of the dorsal horn or from small bundles of axons that penetrated the superficial dorsal horn. These patterns distinguish the VR1-expressing axons from many Aδ afferents that reach the neck of the dorsal horn after arborizing around the lateral edge of the dorsal horn (Light and Perl, 1979). The latter observation is of interest because some studies have suggested that Aδ nociceptive fibers are also sensitive to capsaicin (Nagy et al., 1983).

The pattern of staining in the sacral spinal cord differed considerably from that observed at other spinal levels (Figure 5C). Specifically, here we found extensive axonal staining throughout the dorsal horn. Some fibers

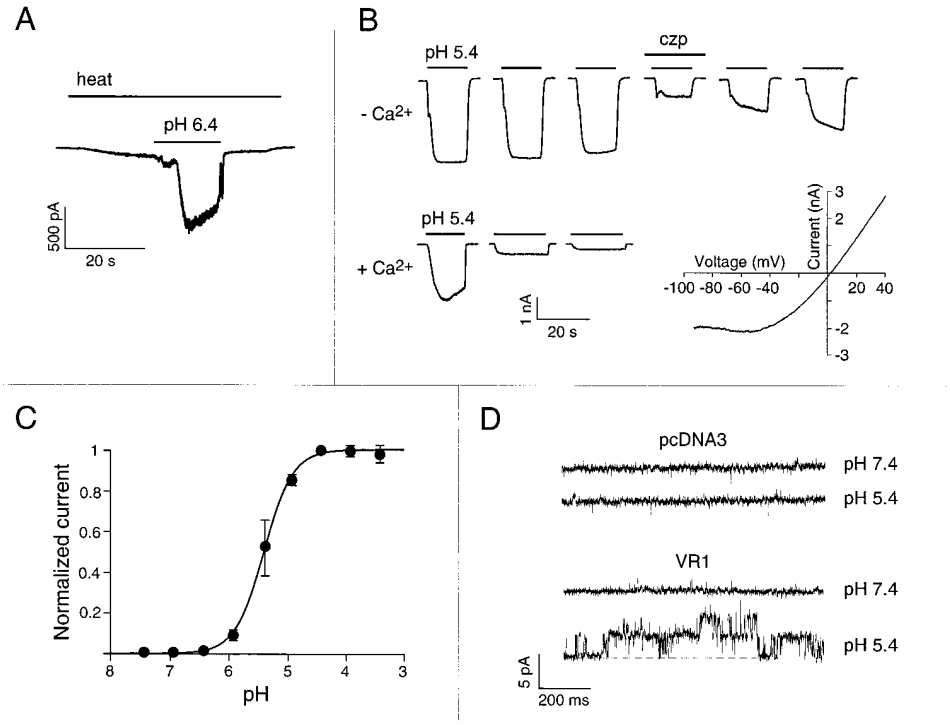

Figure 4. Protons Activate VR1 at Normal Physiological Temperature in HEK 293 Cells

(A) pH 6.4 bath solution activated whole-cell currents in VR1-transfected cells (−60 mV) at 37°C.

(B) High concentrations of protons at 22°C evoked currents in VR1-transfected cells that resemble capsaicin-evoked responses. Under Ca^{2+}-free conditions (top), protons evoked nondesensitizing responses that were reversibly blocked (78.9% ± 4.1%, n = 4) by capsazepine (czp, 10 μM). In Ca^{2+}-containing solution (bottom), proton-evoked currents showed profound desensitization. Whole-cell current–voltage curve generated by a ramp pulse (−100 to +40 mV in 500 ms) showed pronounced outward rectification. Acidic bath solution was applied at 2 min intervals, and pretreatment time with capsazepine was 2 min.

(C) Concentration–response curve for protons shows half-maximal response at pH 5.4. Normalized whole-cell currents (mean ± SEM, n = 12) are expressed as a fraction of pH 4.4 bath solution responses. The Hill equation was used for curve fitting.

(D) Protons (pH 5.4) produce single channel openings in outside-out patches excised from VR1-expressing cells but not vector-transfected cells (E_h = +40 mV). Broken line indicates closed channel level. Mean unitary amplitude was 2.80 ± 0.08 pA (n = 8). No inside-out patches showed responses to acidified bath solution (n = 8). Of these, five were tested for capsaicin responses, and all were positive.

projected to the neck of the dorsal horn, while others could be traced from the dorsal root entry zone to lamina X and thence to the opposite side of the spinal cord. This pattern of staining is reminiscent of the arborization of small diameter pelvic visceral afferents (Morgan et al., 1981). Consistent with this assignment, we observed dense VR1 immunoreactivity in the nucleus of the solitary tract and area postrema, medullary structures that receive vagal projections from visceral organs via the nodose ganglion (Figure 5D). Although we previously failed to detect VR1 messenger RNA within the nodose ganglion (Caterina et al., 1997), the present results clearly demonstrate the expression of this protein within the central targets of vagal afferents. We also observed VR1 immunoreactivity in the vagus nerve, peripheral to the nodose ganglion (data not shown) and in tortuous nerve terminals within muscular and submucosal layers of the bladder (Figure 5E). These findings are consistent with previous reports showing capsaicin sensitivity and [^{3}H]-resiniferatoxin labeling of cells in the nodose ganglion (Szallasi et al., 1995) and marked sensitivity of bladder afferents to capsaicin (Maggi, 1992). VR1 immunoreactivity was also observed in peripheral projections of sensory neurons. Although some receptors can only be detected in the peripheral nerve after ligation (to build up the antigen level at the ligature) (Liu et al., 1994), we found considerable VR1 staining along the course of the sciatic nerve (i.e., very distant from the terminals), suggesting either that the receptor is inserted along the course of the peripheral nerve membrane or that relatively large quantities are transported intraaxonally. By contrast, we found no staining of sympathetic postganglionic neurons, nor did we observe staining in central neurons in the brain, including the preoptic area of the hypothalamus, which reportedly has some resiniferatoxin binding (Acs et al., 1996) and responsiveness to vanilloids (Ritter and Dinh, 1992; Szolcsanyi, 1993).

Electron microscopic (EM) analysis of spinal cord sections revealed VR1 immunoreactivity in large numbers of unmyelinated axons in the dorsal horn (Figure 6A). Importantly, even the rare axon that penetrated into lamina III proved by EM to be unmyelinated. To date, we have not observed labeling of myelinated axons; however, this result could reflect incomplete antibody penetration. VR1 immunoreactivity was also found at nerve terminals, where it was typically concentrated away from synaptic specializations and from regions occupied by collections of clear vesicles (Figure 6B). In some cases, the immunostaining was associated with dense core vesicles, but our use of an enzyme-coupled (DAB)

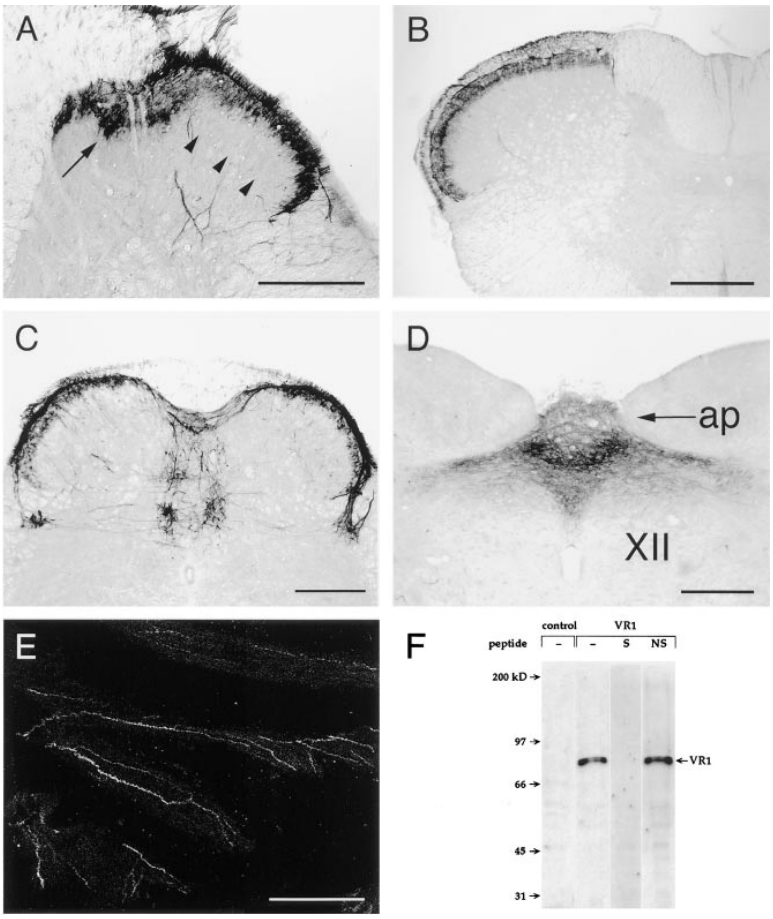

Figure 5. VR1 Is Located in Somatic and Visceral Primary Afferent Neurons

(A–E) Immunoperoxidase staining reveals intense VR1 immunoreactivity in lumbar spinal cord dorsal horn (A), trigeminal nucleus caudalis (B), sacral spinal cord dorsal horn (C), and nucleus of the solitary tract and area postrema (ap) of the caudal medulla (D). Note the characteristic patch of intense VR1 immunoreactivity in the medial part of inner lamina II (arrow), with much less VR1 immunoreactivity in the lateral part of inner lamina II (arrowheads; also see Figure 7E). Immunofluorescence staining reveals VR1 immunoreactivity in axons that arborize in the bladder (E) as well as in the vagus and sciatic nerves (data not shown). Scale bars, 200 μm (A and C); 375 μm (B); 100 μm (D); and 500 μm (E).

(F) Immunoblot analysis reveals a specific 90 kDa band in lysates from VR1-transfected HEK 293 cells but not from pcDNA3-transfected control cells. This band was eliminated by preincubation with the peptide used to generate the VR1 antiserum (S) but not with control peptide (NS). The band was not detected using preimmune serum (data not shown).

detection method did not provide the resolution necessary to establish this point unequivocally.

In the adult rodent, primary afferent nociceptors have been largely divided into two histochemically distinct classes: one expresses neuropeptides (e.g., substance P and calcitonin gene-related peptide [CGRP]); the other expresses specific enzyme markers (e.g., fluoride-resistant acid phosphatase) and binds the lectin IB4. These two classes of neurons are sensitive to the neurotrophic factors nerve growth factor (NGF) and glial cell line–derived neurotrophic factor (GDNF), respectively (Snider and McMahon, 1998). Although sensitivity to neonatal treatment with capsaicin has been observed in both populations (Hammond and Ruda, 1991), the extent to which this is maintained in the adult and mediated through VR1 is unclear. To address these issues, we carried out colocalization studies of IB4 binding and VR1 and substance P immunoreactivity in DRG and spinal cord dorsal horn. In lumbar DRG, we found that most (~85%) of the substance P–immunoreactive cells costained for VR1 (Figure 7A). The much larger population of IB4-positive cell bodies also immunostained for the VR1 but to a lesser extent (60%–80%) (Figure 7B). Finally, we found that ~10% of the VR1-positive neurons stained for neither substance P nor IB4. Similar patterns of costaining were observed in the superficial dorsal horn of the spinal cord (Figure 7E). Here, we found extensive double labeling of substance P and VR1 in lamina I and the outer portion of lamina II, where most peptide-containing afferents terminate. As expected, we found dense IB4 labeling in the inner part of lamina II (IIi), but, interestingly, we found colocalization of dense VR1 immunoreactivity and IB4 binding only in the medial part of IIi. The lateral part of IIi only stained for IB4 (see also Figure 5A). This differential colocalization reveals a heretofore undetected heterogeneity of the small diameter primary afferent termination within the inner portion of lamina II. Alternatively, these axons may derive from DRG neurons that express VR1 but do not target the receptor to the central terminal.

Discussion

VR1 is a Polymodal Signal Detector

We have shown that the cloned capsaicin receptor can be activated by several stimuli, including vanilloid compounds, heat, and protons. Is VR1, by itself, sufficient to mediate responses to these stimuli? The involvement of additional proteins or other cellular components cannot be formally ruled out until active channel protein has been studied in a homogeneous and defined preparation. Nevertheless, because activation or modulation can occur dynamically, in excised membrane patches, and in different cellular environments, we suggest that the ability of VR1 to respond to these dissimilar agonists is an intrinsic property of the channel protein.

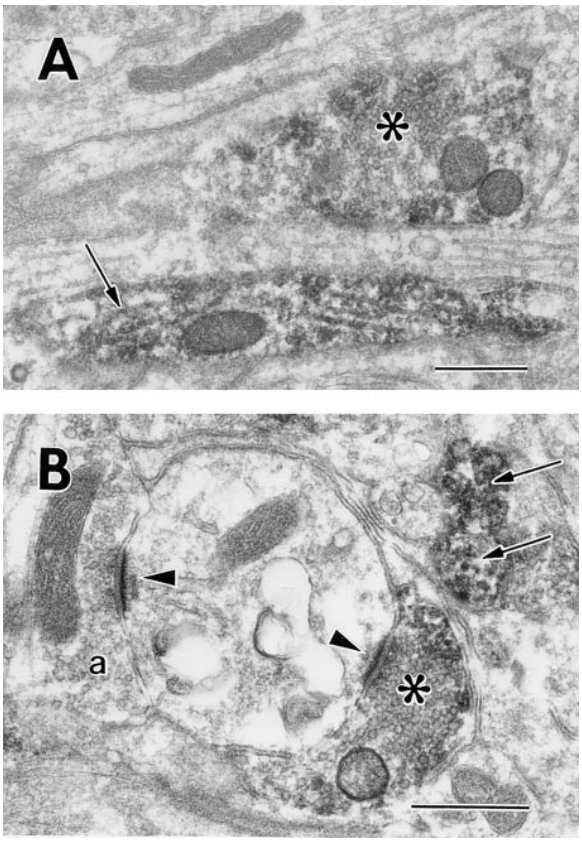

Figure 6. VR1 Immunoreactivity Is Concentrated in Synaptic Terminals in the Dorsal Horn

(A) A sagittal section reveals VR1 immunoreactivity in an unmyelinated axon and a synaptic bouton. Axonal labeling is associated with microtubules (arrow) as well as with the plasma membrane.

(B) VR1 immunoreactivity is seen in a terminal that is presynaptic to a dendrite receiving convergent input from a VR1-negative terminal (a). In the synaptic terminal, VR1 immunoreaction product is often located away from the clusters of clear synaptic vesicles (asterisks in [A] and [B]). The presence of the synaptic density (arrowhead in [B]) makes it difficult to determine whether there is VR1 immunoreactivity at the active zone. Scale bars, 0.2 μm.

How do different VR1 agonists promote channel opening and to what extent do their gating mechanisms overlap? It appears that heat, protons, and vanilloids do not interact with the channel in completely equivalent ways. For example, although both capsaicin and proton-induced desensitization are calcium dependent, heat-induced desensitization is independent of extracellular calcium. In addition, the ability of heat and capsaicin to overcome desensitization when applied together, but not separately, suggests that the effects of these agonists on the channel differ. Together, these findings argue against a simple model in which all three agonists act at one site to gate channel opening through a single conserved mechanism. Perhaps this is to be expected, since heat and protons, in contrast with drugs, will have global effects on protein conformation and membrane properties that could influence the nature of ion channel responses to these stimuli. On the other hand, because capsazepine blocks capsaicin-, heat-, and proton-evoked currents, there is clearly some degree of convergence in the mechanisms of action of these three stimuli. Assuming that capsazepine blocks responses to all three agonists via the same mechanism, this observation rules out another extreme model wherein completely nonconvergent pathways lead to channel activation.

An alternative and particularly intriguing model is one in which capsaicin binding alters the sensitivity of VR1 to temperature and acidity such that the channel opens under normal physiological conditions. In this scenario, capsaicin is not an agonist per se but functions as a modulatory agent, lowering the channel's response threshold to the ubiquitous actions of heat and protons. Precedence for such a mechanism is provided by two other lipid-soluble plant-derived toxins, aconitine and veratridine. These alkaloid compounds act as "agonists" for voltage-gated sodium channels by altering their voltage dependence so that the channel opens at resting membrane potential (Strichartz et al., 1987; Hille, 1992). Interestingly, aconitine and veratridine reduce the strong preference that voltage-gated Na^+ channels exhibit for Na^+ ions, a phenomenon that resembles the quantitative differences we observe in the relative ionic permeability ratios of heat- and capsaicin-evoked responses. This model highlights the notion that vanilloids, heat, and protons act in concert to regulate VR1 activity; the effects of any one stimulus cannot be considered in isolation. Mutational analysis of VR1 function and the development of stimulus-specific VR1 antagonists will help to distinguish among these and other potential mechanisms of channel activation.

VR1 As a Mediator of Sustained Proton Responses In Vivo

In sensory neurons, proton-evoked currents consist of two major components: one is rapidly inactivating and Na^+ selective with a linear current–voltage relation (Krishtal and Pidoplichko, 1980; Konnerth et al., 1987); the other is a more sustained, nonselective cation conductance with an outwardly rectifying current–voltage profile (Bevan and Yeats, 1991; Bevan and Docherty, 1993; Bevan and Geppetti, 1994). The latter component is believed to underlie the prolonged sensation of pain that accompanies tissue acidification. Although it has been proposed that DRASIC, a member of the degenerin/amiloride-sensitive cation channel family (Waldmann et al., 1997b) mediates the sustained phase of proton-evoked responses in sensory neurons, the pH dependence of the sustained component of DRASIC-mediated currents (half-maximal effective $pH_o = 3.5$) is significantly lower than that of sustained proton-evoked currents in sensory neurons. VR1, by contrast, is activated by protons in a concentration range that more closely resembles that associated with sustained proton-evoked currents in sensory neurons (Bevan and Yeats, 1991; Kress et al., 1996), proton-stimulated peptide release from innervated tissue (Geppetti et al., 1990; Bevan and Geppetti, 1994), and ischemic and inflammatory models of tissue injury (Jacobus et al., 1977; Steen et al., 1992; Bevan and Geppetti, 1994). Despite this similarity, it is still uncertain whether VR1 can fully account for the pharmacological properties of sustained

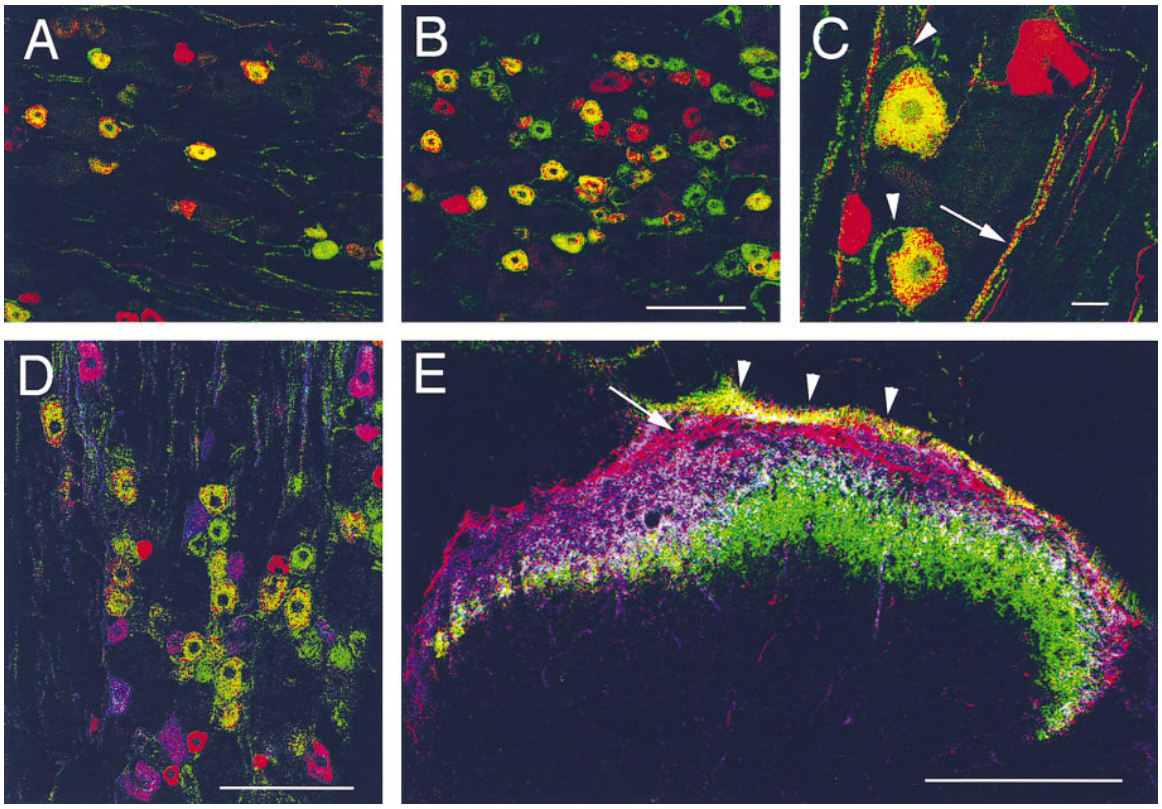

Figure 7. VR1 Is Expressed in Both Peptide-Containing and Nonpeptide Primary Afferent Neurons

Double immunofluorescence was used to colocalize VR1 (red) and substance P (green) immunoreactivity (A and C) or VR1 immunoreactivity (red) and lectin IB4 binding (green) (B) in DRG cell bodies. The great majority of substance P–immunoreactive cell bodies also express VR1 (yellow in [A] and [C]); however, although many of the axons are also double labeled (arrow in [C]), some axons of substance P/VR1-positive neurons were only substance P positive (arrowheads in [C]). Numerous IB4/VR1-positive cell bodies were found in the DRG, but many IB4-positive neurons do not express VR1 (green cells in [B]). Triple labeling for VR1 (red), substance P (blue), and IB4 (green) in the DRG (D) reveals the presence of VR1-expressing neurons that are neither substance P nor IB4 positive. Triple labeling in the dorsal horn (E) reveals extensive terminal overlap of VR1 and substance P in laminae I and outer II (purple). There appears to be some VR1-only labeling in lamina I (arrow). Intense double labeling of VR1 and IB4 is seen in axons of the Lissauer tract (arrowheads) and in the medial part of inner lamina II (yellow); however, there is also a dense concentration of IB4-only axons (green) in the lateral part of inner lamina II. Scale bars, 50 μm (B, D, and E); 10 μm (C).

proton-evoked pain responses. Most notably, although some studies have reported capsazepine block of proton-evoked responses in sensory neurons (Santicioli et al., 1993; Liu and Simon, 1994; Fox et al., 1995), others have not (Bevan et al., 1992; Bevan and Geppetti, 1994; Fox et al., 1995). These discrepancies could reflect functional redundancy of proton-sensing molecules, differences in experimental protocols, or nonselective and noncompetitive mechanisms of capsazepine action (Liu and Simon, 1997). Nevertheless, VR1 remains a viable candidate for a transducer of proton-evoked responses in the "pain" pathway.

VR1 Localization and Nociceptor Heterogeneity

A recent review on the molecular biology of nociceptors raised questions regarding the expression of VR1 in peptide-containing, NGF-sensitive sensory neurons versus IB4-binding, GDNF-sensitive neurons (Snider and McMahon, 1998). Our demonstration of VR1 immunoreactivity within substance P–positive and IB4-positive populations suggests that VR1 can account for the capsaicin sensitivity of both cell types. At the same time, our finding that many IB4-positive cells were not VR1 positive provides evidence for the existence of two neurochemically distinct populations of IB4-binding nociceptors that project to anatomically discrete regions of lamina II of the spinal cord dorsal horn. Because the medial and lateral regions of the lumbar dorsal horn represent distal and proximal parts of the hindlimb, respectively (Devor and Claman, 1980), this result further suggests that functionally distinct afferent subtypes innervate topographically different regions of the hindlimb. Finally, these results are consistent with there being a subpopulation of IB4-positive cells that is either resistant to capsaicin action or sensitive by virtue of another receptor subtype. Given that VR1 responds to thermal and chemical stimuli and that the traditional polymodal nociceptor also responds to noxious mechanical stimuli (Bessou and Perl, 1969), it will be of great interest to determine whether mechanical sensitivity is differentially manifest within the nociceptor populations described.

Polymodal Activation of VR1 In Vivo

The relative importance of noxious thermal and chemical stimuli as activators of VR1 in vivo is likely to vary with

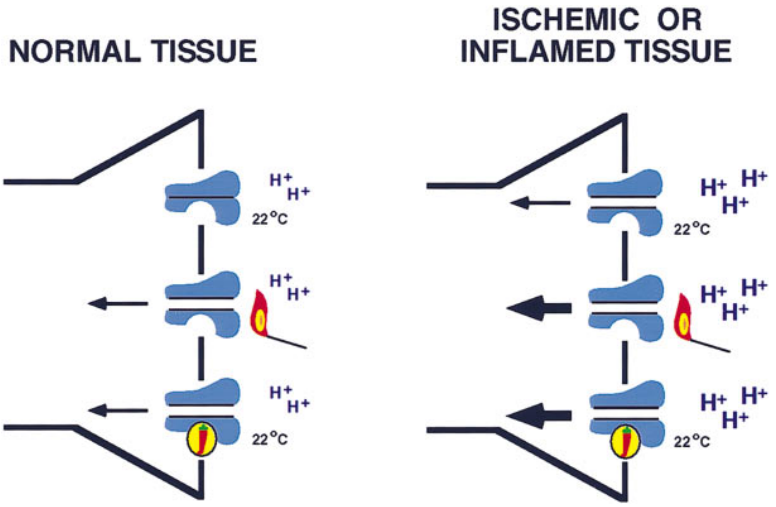

Figure 8. Proposed Model for VR1 Integration of Painful Stimuli in Normal and Ischemic or Inflamed Tissues

Heat (flame) or capsaicin (pepper) can evoke pain in normal animals by activating VR1 in nociceptor terminals at physiological pH (left). In ischemic and inflamed tissue (right), we hypothesize that VR1 is activated at room temperature by the elevated proton (H^+) concentrations that these insults produce. In addition, protons potentiate responses evoked by heat or capsaicin, resulting in increased nociceptor activity. Thickness of arrows corresponds to relative magnitude of nociceptor activity, which in turn contributes to the magnitude of the resultant pain response.

the anatomical location and nature of the insult. Thus, in superficial (e.g., cutaneous) tissues VR1 activation might result from acute elevations in temperature or reductions in pH, whereas in visceral or deep somatic tissues (where temperatures are unlikely to reach the "noxious" level of 43°C) changes in pH are more likely to drive VR1 activation. These scenarios do not preclude the existence of other modulators of VR1 activity, such as novel VR1 agonists or proalgesic agents (e.g., bradykinin and prostaglandins) that could alter vanilloid receptor function indirectly via second messenger signaling pathways (Levine and Taiwo, 1994). Moreover, channels other than VR1 may contribute to the thermal and chemical sensitivity of primary afferent neurons.

Following tissue injury, the magnitude of VR1 responses is likely to reflect the combined effects of protons and temperature (Figure 8). We have shown that in the context of a normal ambient thermal stimulus (i.e., 37°C) moderate reductions in pH can activate VR1. This interaction would be expected to facilitate pain responses and/or autonomic reflexes during relatively mild acidosis in ischemic or inflamed tissues. Reductions in pH that accompany tissue injury would also be expected to enhance the responsiveness of those tissues to exogenously applied thermal stimuli. In fact, the augmentation of VR1 thermal responsiveness by protons shows a striking resemblance to the increase in cutaneous nociceptor thermal sensitivity associated with inflammation (Fields, 1987; Dubner and Basbaum, 1994; Meyer et al., 1994). 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. Given these parallels, modulation of VR1 activity may serve as a useful model system with which to analyze the molecular basis of the sensitization of nociceptors that contributes to the development of allodynia and hyperalgesia.

Experimental Procedures

Mammalian Cell Electrophysiology

Patch-clamp recordings were carried out with VR1-transfected HEK 293 cells in whole-cell, inside-out, and outside-out configurations as previously described (Caterina et al., 1997). Standard bath solution for whole-cell recordings contained (in mM) 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose (adjusted to pH 7.4 with NaOH). In Ca^{2+}-free bath solution, $CaCl_2$ was replaced with 5 mM EGTA. Bath solution was buffered to different pH values with either 10 mM HEPES (6.9 and 6.4) or 10 mM MES (5.9, 5.4, 4.9, 4.4, 3.9, and 3.4). Ca^{2+}-free bath solution was used unless otherwise stated. For divalent cation substitution experiments, whole-cell configuration was obtained in standard bath solution, after which the solution was changed to (in mM) 140 NaCl, 10 glucose, and 10 HEPES (adjusted to pH 7.4 with NaOH) followed by 110 $MgCl_2$ (or $CaCl_2$), 2 $Mg(OH)_2$ (or $Ca[OH]_2$), 10 glucose, and 10 HEPES (adjusted to pH 7.4 with HCl). Reversal potential was measured using voltage ramps (−100 to +40 mV in 500 ms). Bath solution for outside-out patch recordings and pipette solution for inside-out patch recordings contained (in mM) 140 NaCl and 10 HEPES (adjusted to pH 7.4 with NaOH). Bath solution for inside-out patch recordings and pipette solutions for outside-out patch recordings and cation substitution experiments contained (in mM) 140 NaCl, 10 HEPES, and 5 EGTA (adjusted to pH 7.4 with NaOH). Pipette solution for other whole-cell recordings contained (in mM) 140 CsCl (or 130 Cs aspartate and 10 NaCl for obtaining the current–voltage curve), 5 EGTA and 10 HEPES (adjusted to pH 7.4 with CsOH). Liquid junction potentials were measured directly in separate experiments and did not exceed 3 mV with solutions used at 22°C. In heat experiments, significant liquid junction potential changes (>6 mV) were observed and corrections made for membrane potentials and reversal potentials. Whole-cell recording data were sampled at 20 kHz and filtered at 5 kHz for analysis (Axopatch 200 amplifier with pCLAMP software, Axon Instruments). Single channel recording data were sampled at 10 kHz and filtered at 2 kHz. Permeability ratios for divalent cations to Na^+ (P_x/P_{Na}) were calculated as previously described (Caterina et al., 1997). Linear and nonlinear regression analyses were conducted using Origin (Microcal) or DeltaGraph (Delta Point).

Oocyte Electrophysiology

Defolliculated *Xenopus laevis* oocytes were injected with 5–10 ng VR1 cRNA as previously described (Caterina et al., 1997). Two-electrode voltage-clamp analysis (E_h = −40 mV) was carried out 4–7 days postinjection. Frog Ringer's solution contained (in mM) 90 NaCl, 1.0 KCl, 2.4 $NaHCO_3$, 0.1 $BaCl_2$, 1.0 $MgCl_2$, and 10 HEPES (at pH 7.6, unless otherwise indicated). Bath solution temperature was controlled with an in-line SH-27A solution heater and TC-324B thermal controller (Warner Instruments) and was accurate to within 0.1°C between 40°C and 50°C. Below 40°C, accuracy decreased with decreasing temperature to a maximum deviation of 1.1°C at 30°C and 2.7°C at 22°C. A thermistor was placed within 1 mm of the oocyte to record bath temperatures and regulate the heat controller.

Immunolocalization of VR1

A peptide encoding the predicted carboxyl terminus of VR1 (EDAEV FKDSMVPGEK) was coupled to keyhole limpet hemocyanin via an

amino-terminal cysteine and used to immunize rabbits (HTI Bioproducts). Crude serum or ammonium sulfate–purified material was used at specified dilutions (Harlow and Lane, 1988). For immunoblot analysis, HEK 293 cells were transiently transfected with 24 μg control vector (pcDNA3) or VR1–pcDNA3, as described (Caterina et al., 1997). After 16 hr, cells were washed with phosphate-buffered saline/1 mM EDTA, lysed with Laemmli sample buffer, and incubated at 55°C for 10 min. Then, 0.5% of this sample was subjected to SDS-PAGE on a 7.5% Laemmli gel, blotted to a nitrocellulose membrane, and probed with anti-VR1 serum (diluted 1000-fold) in the absence or presence of VR1 carboxy-terminal peptide (5 μg/ml). Immunodetection was performed using the enhanced chemiluminescence (ECL) Western blotting kit (Amersham).

For light microscopic studies, male C57BL/6 mice (23–28 g) or male Sprague-Dawley rats (250–300 g) (Bantin-Kingman Laboratories) were deeply anesthetized with 100 mg/kg sodium pentobarbital administered intraperitoneally and perfused through the ascending aorta with 0.1 M phosphate-buffered saline followed by a 10% formalin or 4% paraformaldehyde fixative. After perfusion, tissues were removed, postfixed for 4 hr in the same fixative, and then cryoprotected in 30% sucrose overnight at 4°C. The spinal cord was sectioned transversely at 30 μm on a freezing microtome; DRG, sciatic nerve, and superior cervical ganglion sections (10 μm) and bladder and vagus nerve sections (25 μm) were cut on a cryostat. For immunocytochemistry, sections were incubated overnight in anti-VR1 antiserum diluted 1:50,000. Immunostaining was performed according to the avidin–biotin peroxidase method (Hsu et al., 1981) using a nickel-intensified diaminobenzidine protocol with glucose oxidase to localize the horseradish peroxidase immunoreaction product. Preincubation of the primary antiserum with 10 μg/ml of the VR1 carboxy-terminal peptide abolished staining (data not shown). Reacted sections were mounted on gelatin-coated slides, dried, dehydrated, and then coverslipped with Eukitt (Calibrated Instruments). Sections were examined with a Nikon Microphot-FXA microscope and digitized images obtained with a MicroLumina camera (Leaf Systems); photomontages were created with Adobe Photoshop. To localize VR1 in bladder, sciatic nerve, and vagus nerve, sections were incubated overnight (4°C) with VR1 antiserum (diluted 1:5,000–1:7,500), washed, incubated 2 hr with Cy3-conjugated goat anti-rabbit IgG (Jackson Immunochemicals, diluted 1:600), washed, dehydrated, and mounted in DPX (Electron Microscopy Sciences). Preincubation of the primary antiserum with 10 μg/ml of the VR1 carboxy-terminal peptide abolished immunofluorescent staining, whereas preincubation with a control peptide did not (data not shown). To double label DRG cells for VR1 and substance P, we incubated tissue overnight in the rabbit VR1 antiserum diluted 1:5,000 and in a guinea pig anti–substance P antiserum diluted 1:15,000 (kindly provided by Dr. John Maggio). The two primary antisera were respectively localized with a Cy3-coupled goat-anti rabbit IgG diluted 1:200 and a Cy2-coupled goat anti–guinea pig IgG diluted 1:600 (both from Jackson Immunochemicals). For costaining with *Griffonia simplicifolia* IB4 lectin, sections were incubated with 12.5 μg/ml FITC-conjugated IB4 lectin (Sigma) and then with VR1 antiserum using a Cy3-conjugated secondary antiserum. Reacted sections were examined with a BioRad 1024 confocal microscope (Bio-Rad). Confocal images were transferred to NIH image and then analyzed with Adobe Photoshop.

For electron microscopy, adult rats were perfused with 4% formaldehyde and 1% glutaraldehyde in phosphate buffer, and spinal cords were sectioned on a Vibratome. After treatment of sections with 50% ethanol to enhance penetration of antibodies, we immunostained for VR1 at the EM level as previously described (Llewellyn-Smith and Minson, 1992). Immunoreacted sections were then osmicated, embedded in plastic, and thin sectioned for examination in a JEOL electron microscope.

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