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Molecular Basis for Species-Specific Sensitivity to "Hot" Chili Peppers

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Summary

Chili peppers produce the pungent vanilloid compound capsaicin, which offers protection from predatory mammals. Birds are indifferent to the pain-producing effects of capsaicin and therefore serve as vectors for seed dispersal. Here, we determine the molecular basis for this species-specific behavioral response by identifying a domain of the rat vanilloid receptor that confers sensitivity to capsaicin to the normally insensitive chicken ortholog. Like its mammalian counterpart, the chicken receptor is activated by heat or protons, consistent with the fact that both mammals and birds detect noxious heat and experience thermal hypersensitivity. Our findings provide a molecular basis for the ecological phenomenon of directed deterrence and suggest that the capacity to detect capsaicin-like inflammatory substances is a recent acquisition of mammalian vanilloid receptors.

Introduction

Capsaicin and other pungent vanilloid compounds evoke a sensation of tingling and burning pain by activating a nonselective cation channel, called VR1, on sensory nerve endings (Caterina et al., 1997; Julius and Basbaum, 2001). Electrophysiological and genetic studies have shown that VR1 is also activated by heat (>43°C) and contributes to the detection of noxious thermal stimuli by primary sensory neurons of the pain pathway (Caterina et al., 1997; Tominaga et al., 1998; Caterina and Julius, 2001). Moreover, VR1 is sensitized by a number of chemical factors produced during inflammation, some of which (e.g., extracellular protons and lipid metabolites) interact with the channel directly to potentiate the effects of capsaicin or heat (Tominaga et al., 1998; Jordt et al., 2000; Sprague et al., 2001). Indeed, VR1-deficient mice do not display thermal hypersensitivity following tissue injury (Caterina et al., 2000; Davis et al., 2000), substantiating the idea that the capsaicin receptor is a polymodal integrator of noxious chemical and physical stimuli in vivo. Because capsaicin is structurally related to putative endogenous VR1 agonists, such as anandamide (Zygmunt et al., 1999) and 12-HPETE (Hwang et al., 2000), there is significant pharmacological and physiological interest in identifying regions of the channel that transduce the effects of these molecules. VR1 is a member of the greater family of TRP cation channels (Clapham et al., 2001), and thus identification of a vanilloid site on VR1 may also provide more general information about the mechanism whereby TRP channels are regulated by endogenous lipids, such as diacylglycerol, arachidonic acid, or PIP_2 (Chyb et al., 1999; Hofmann et al., 1999; Chuang et al., 2001; Estacion et al., 2001; Hardie and Raghu, 2001).

In the present study, we describe the cloning of an avian vanilloid receptor ortholog from chicken sensory neurons. While functioning as a polymodal integrator for noxious stimuli, the avian channel fails to be activated by capsaicin, thereby providing a molecular explanation for insensitivity of birds to "hot" chili peppers. In our molecular analysis of this phenomenon, we map the structural determinants for vanilloid interaction to a small segment within the transmembrane moiety of the receptor. This domain is sufficient to confer all pharmacological and functional aspects of vanilloid actions to the avian channel and to two more distantly related capsaicin-insensitive mammalian homologs, VRL1 and OTRPC4 (VR-OAC). Finally, we find that this segment determines the receptor's sensitivity to the endogenous agonist (endovanilloid), anandamide.

Results

The Avian Capsaicin Receptor Ortholog Is an Integrator of Noxious Stimuli with Rudimentary Vanilloid Sensitivity

Primary sensory neurons from chicks are capsaicin-insensitive (Wood et al., 1988). However, they do exhibit heat-evoked membrane currents resembling those carried by mammalian vanilloid receptors, both of which are characterized by steep outward rectification, a temperature threshold of ~45°C, and blockade by the noncompetitive vanilloid receptor antagonist, ruthenium red (Marín-Burgin et al., 2000; Nagy and Rang, 2000). These observations suggest that birds may express a vanilloid-insensitive homolog of rVR1.

To test this hypothesis, we screened a chick dorsal root ganglion (DRG) cDNA library with a rat vanilloid receptor (rVR1) probe and identified a clone (cVR1) whose predicted protein sequence shows 68% identity and 79% similarity to rVR1 (Figure 1A). Functional analysis in *Xenopus* oocytes revealed that cVR1 is activated by ambient temperature >45°C, and, like rVR1, this response is markedly potentiated (~5-fold) by extracellular acidification to pH 6.3 (Figures 1B and 1C). Moreover, the chick channel could be directly activated by acidic (pH 4) extracellular solution (Figure 1D), and this response was blocked by ruthenium red (Figure 1E). In contrast, capsaicin produced no detectable currents, even at a concentration (100 μM) exceeding the EC_{50} for rVR1 by 200-fold (Figure 1D). However, two observations suggest that cVR1 may contain rudimentary elements of a vanilloid-binding site. First, perfusion of cVR1-expressing oocytes with a high dose (50 μM) of capsaicin produced a small and slowly developing enhancement of proton (pH 5.5)-evoked currents (Figure 2A). Second, pH 4.0-evoked currents were partially blocked by 20 μM capsazepine (Figure 2B), a competitive capsa-

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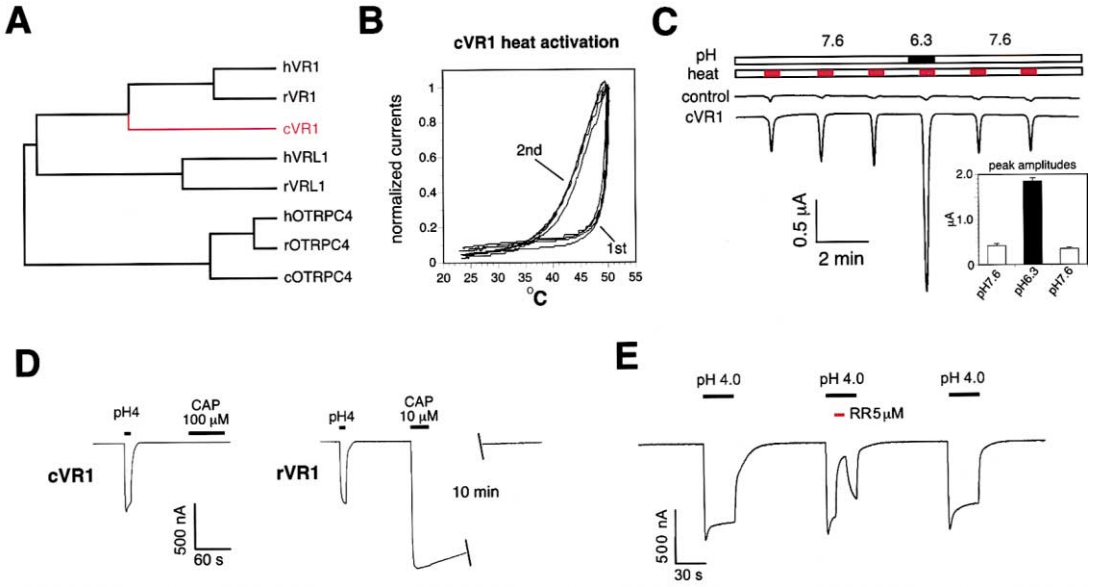

Figure 1. The Chicken VR1 Ortholog Responds to Noxious Heat or Protons, but Not to Capsaicin

(A) Phylogenetic tree based on CLUSTALW alignment of vanilloid receptor-related ion channel protein sequences (VR1, VRL1, and OTRPC4) from human, rat, and chicken.

(B) cVR1 channels were expressed in *Xenopus* oocytes and activated by heating of perfusate from room temperature to 50°C within 20 s. Individual currents recorded from 4 independent voltage-clamped (−40 mV) oocytes were normalized to peak current at 50°C. At the first heat application, cVR1 currents activated with a threshold of ~45°C. Responses to a second heat pulse showed marked sensitization, as observed with rVR1.

(C) Heat-evoked currents were produced in voltage-clamped oocytes expressing cVR1 by raising the bath temperature to 48°C within 15 s. This procedure was repeated six times in 2 min intervals. During the fourth heat pulse, the bath pH was decreased from 7.6 to 6.3, producing strong potentiation of the current response. Water-injected oocytes served as control. Inset shows average peak currents for the third (pH 7.6), fourth (pH 6.3), and fifth (pH 7.6) heat application. Heat-activated currents were potentiated 470% ± 60% (n = 4) at pH 6.3. Error bars represent SEM.

(D) Currents were activated by perfusion of a cVR1-expressing oocyte (left) with acidic (pH 4.0) solution for 10 s. Average peak currents at pH 4.0 were 600 ± 60 nA (n = 6) after 4 days of expression. Subsequent perfusion with capsaicin (100 μM) for 1 min produced no detectable current. In contrast, rat VR1 (right) was robustly activated by extracellular protons or capsaicin (10 μM).

(E) Ruthenium red block of proton-evoked currents in cVR1-expressing oocytes. The pH of the extracellular solution was lowered from 7.6 to 4.0 for 30 s. At the second application, ruthenium red (5 μM) was coapplied for 10 s while the extracellular solution was kept at pH 4.0. The average block of proton-activated currents after 10 s was 85% ± 3% (n = 6). Ruthenium red block is reversible.

icin antagonist that has also been shown to attenuate native heat-evoked responses in chick DRG neurons (Marín-Burgin et al., 2000). We also tested the sensitivity of cVR1 to methylanthranilate, a noxious repellent that is specific for birds and which has been used in conditioning experiments (Kare, 1961; Lee-Teng and Sherman, 1966; Richard and Davies, 2000). cVR1 did not respond to this compound (20 μM, n = 5, not shown), suggesting that it acts on a different target in avian sensory neurons. In summary, our analysis demonstrates that, with the exception of its significantly reduced capsaicin sensitivity, cVR1 has functional properties similar to those of its mammalian counterpart and entirely consistent with the known physiological and behavioral responses of birds to capsaicin or noxious heat. Thus, based on both genetic and functional criteria, we conclude that cVR1 is the ortholog of the mammalian vanilloid receptor.

Determinants of Vanilloid Sensitivity Reside within a Transmembrane Segment of the Capsaicin Receptor

Sequence differences between cVR1 and rVR1 are peppered throughout the protein, making it impossible to guess which region(s) might confer vanilloid sensitivity to the latter. To identify such a domain, we generated a series of chimeric ion channels between rVR1 and cVR1, as well as with two capsaicin-insensitive mammalian homologs, the heat-activated channel VRL1 (48% identity) (Caterina et al., 1999), and the osmosensitive channel OTRPC4 (or VR-OAC, 42% identity) (Liedtke et al., 2000; Strotmann et al., 2000) (Figure 3A). Strikingly, we found that transfer of a single minimal region spanning transmembrane domains 2 through 4 of rVR1 was sufficient to confer binding of the high affinity vanilloid radioligand, ^{3}H-resiniferatoxin (RTX), to all three channels. Whereas wild-type channels showed no specific binding, the affinity and cooperativity of ^{3}H-RTX binding by the VRL1-based chimera (V2-4/L) were nearly identical to that of rVR1 (K_D = 270 ± 10 pM; n_{Hill} = 1.7 ± 0.2 versus 200 ± 30 pM; n_{Hill} = 1.6 ± 0.2) (Figure 3B). The OTRPC4-based chimera V2-4/O also showed robust ligand binding (Figure 3C). Similarly, transfer of the TM2-4 region to cVR1 (V2-4/C) resulted in a 3.5-fold ± 0.2 -fold increase of binding over background (20 μg/ml protein; 1nM ^{3}H-RTX; n = 4).

Although vanilloid binding in all three chimeras was equally robust, we observed differences in channel gat-

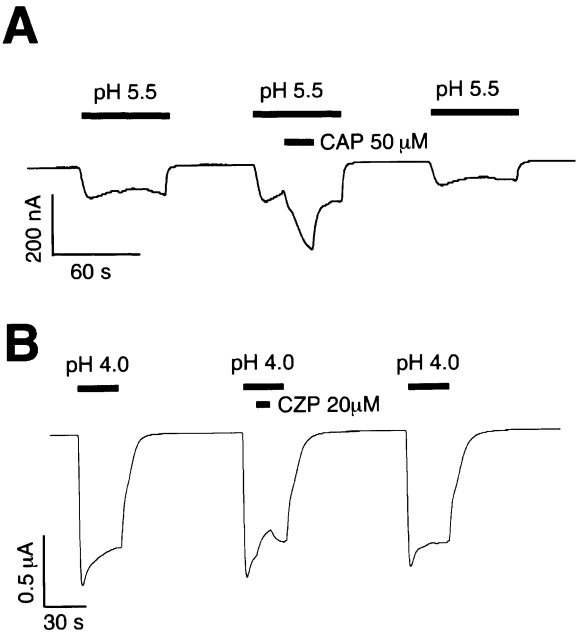

Figure 2. Residual Vanilloid Sensitivity of cVR1

(A) Capsaicin enhances cVR1 proton-evoked currents at high concentrations. An oocyte expressing cVR1 was perfused with pH 5.5 bath solution for 60 s. During the second application, capsaicin was added to a final concentration of 50 μM. A slow and reversible potentiation of proton-activated current was observed.

(B) Weak block of proton-evoked cVR1 currents by capsazepine. cVR1 currents were activated by perfusion of the oocyte with acidic (pH 4) bath solution for 30 s. At the second application, capsazepine (CZP) was added at a concentration of 20 μM. A weak block of proton-activated currents was observed.

ing. This phenomenon correlated with the degree of structural relatedness among the channels. Thus, while V2-4/L contained an intact ^{3}H-RTX binding site, it showed relatively small inward current responses to high doses of capsaicin (50 ± 20 nA, 20 μM capsaicin; n = 5) (Figure 3B, inset). However, in the case of the avian ortholog, transfer of the TM2-4 region was sufficient to confer not only ^{3}H-RTX binding, but also robust capsaicin-mediated channel activation. Expression of this chimera (V2-4/C) in oocytes resulted in large, capsaicin-evoked, inward currents with an EC_{50} of 1.5 ± 0.1 μM (Hill coefficient = 2.0 ± 0.1, n = 5), only ~5-fold higher than that of the rat receptor. V2-4/C was also activated by the synthetic vanilloid agonist, olvanil, and blocked by capsazepine (Figure 3D). We could further limit the essential domain to a cluster of residues located in and adjacent to TM3. The resulting chimera (V3/C) responded with robust inward currents to capsaicin, and as observed with mammalian VR1, these currents were potentiated by extracellular protons (Figure 3E). Taken together, our results indicate that species-specific sensitivity to capsaicin can be ascribed to ~8 amino acids in the vicinity of TM3 that differ between cVR1 and mammalian (rat and human) orthologs (Figure 3F).

Residues Essential for Vanilloid Binding Are Accessible from the Intracellular Side

How might vanilloid compounds associate with this region of the channel? Some insight may be gleaned from the recently solved crystal structure of capsaicin bound to the quinone (Q_B) site of a bacterial photosynthetic reaction center (RC) (Spyridaki et al., 2000). Capsaicin has been found to inhibit photosynthetic activity with an IC_{50} of ~10 μM by replacing Q_B from its binding site at the interface between the aqueous medium and the membrane. Within the RC, the ligand is contacted by 6 residues distributed over a stretch of ~40 amino acids. A phenylalanine located at the cytosol-bilayer interface appears to play a critical role in this binding pocket, where it stacks with the aromatic vanillyl moiety via π-electron interactions. The polar moiety of capsaicin is further stabilized by hydrogen bonds, and its lipophilic tail interacts with several hydrophobic residues within the membrane. Interestingly, the TM3 domain of VR1 is comparable in size to the RC binding region. With this analogy in mind, we used mutagenesis and chemical modification methods to identify subregions or residues within the TM2-4 domain that are important for vanilloid interaction. Because vanilloid compounds are likely to act as allosteric ligands, we expected to find two main classes of mutations: some that specifically alter vanilloid sensitivity, and others that affect channel activation by all stimuli, including protons and heat.

Because cVR1 may have a rudimentary vanilloid binding site, we examined a series of rVR1-VRL1 chimeras to identify residues that contribute to vanilloid sensitivity, but which might be conserved in the avian receptor. We first analyzed chimeric channels in which nine non-overlapping segments from the TM2-4 region of rVR1 were replaced by cognate segments from rVRL1 (Figure 4A). Expression of these mini chimeras in transfected HEK293 cells was confirmed by Western blot analysis (not shown). All substitutions within the hydrophobic transmembrane regions eliminated ^{3}H-RTX binding at a concentration of 500 pM, but some chimeras remained sensitive to high concentrations of capsaicin (20 μM) (Figure 4A). However, substitution of either of two discontinuous regions affecting the inner segments of TM2 or TM3 eliminated all vestiges of ^{3}H-RTX binding and capsaicin-evoked gating (Figure 4A), confirming the importance of TM3 and consistent with the idea that a region(s) of the channel closest to the cytoplasmic leaflet of the bilayer is most important for capsaicin sensitivity.

We therefore analyzed 20 residues within this region, addressing both differences with VRL1 and the more similar chick receptor. While several mutations in TM3 caused minor changes in receptor activation (Figure 4B), substitutions at two positions (R491G in TM2 and S512F in TM3) resulted in a dramatic reduction in both proton and capsaicin-evoked currents (<200 nA with 10 μM capsaicin, n = 4 each). However, introduction of other mutations at these positions (S512A, S512T, or R491E) resulted in a greater preservation of functionality, and in each case, we observed a greater reduction in capsaicin sensitivity compared to extracellular protons (Figures 4C and 4D). Mutant S512Y showed little response to capsaicin up to 100 μM, and no detectable specific binding of resiniferatoxin (not shown). Nevertheless, proton sensitivity was retained (58% ± 8% of wt currents at pH 4.0; Figures 4C and 4D), as were responses to noxious heat, albeit with a slightly higher thermal threshold (>48°C) and smaller maximal amplitudes compared to wild-type receptors (not shown). Similar to the wild-

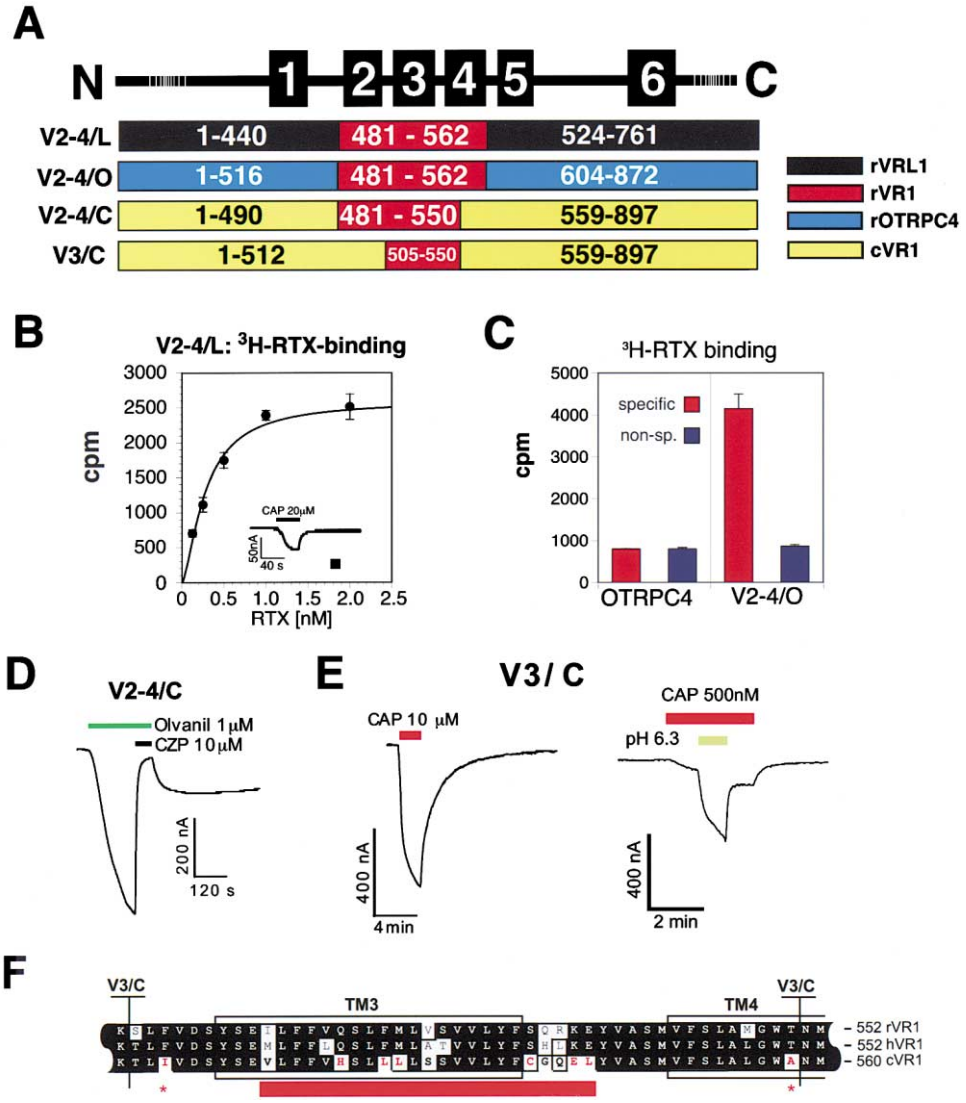

Figure 3. Vanilloid Sensitivity Is Transferred to VR1-Related Homologs through Exchange of Subdomains Containing Transmembrane Regions 2–4

(A) Representative vanilloid-sensitive chimeras containing segments of VR1 (red), VRL1 (black), OTRPC4 (blue), or cVR1 (yellow). In chimera V2-4/L (top), TM2-4 of VR1 replaces the corresponding segment of VRL1. Within this 82 amino acid-long domain, VRL1 and VR1 show identity at 36 positions (43%) and similarity at 16 positions (59% similarity overall). In chimera V2-4/O, these segments of VR1 and OTRPC4 show the same degree of sequence similarity. In cVR1, the essential domain could be reduced to a small segment encompassing TM3 (chimera V3/C).

(B) Purified membranes from HEK293t cells transfected with chimera V2-4/L (2.5 μg/ml total membrane protein) show saturable and specific binding of the vanilloid receptor radioligand ^{3}H-RTX (K_D = 270 ± 10 pM; B_{max} = 440 ± 40 fmol/mg membrane protein (n = 5). Inset: Oocytes injected with V2-4/L cRNA show small current responses to high concentrations of capsaicin.

(C) Chimera V2-4/O showed specific ^{3}H-RTX binding, whereas the parent channel, OTRPC4, did not (500 pM ^{3}H-RTX; 1.5 μg/ml protein; n = 3 each).

(D) Chimera V2-4/C, based on cVR1, responds to olvanil (1 μM), a synthetic vanilloid agonist, and is blocked by capsazepine (10 μM), a competitive capsaicin antagonist. Block by capsazepine appears to wash out rapidly, while activation by olvanil persists over a longer period.

(E) Activation of chimera V3/C, containing the minimal domain conferring vanilloid sensitivity, by capsaicin. Left: Chimeric channels are strongly activated by 10 μM capsaicin. Right: Similar to rat VR1, capsaicin (500 nM)-activated currents in the chimera V3/L are potentiated at lower pH (6.3).

(F) Molecular determinants of species-specific vanilloid sensitivity. Sequence alignment of rat (top), human (middle), and chicken (bottom) VR1 within the TM3-4 region is shown. Conserved residues are indicated by black background. The chimera V3/C contains a minimal segment of rat VR1 that is sufficient to confer vanilloid sensitivity. Within that segment, cVR1 differs from both rat and human VR1 at eight unique positions (shown in red) that are mostly clustered in and around TM3 (red bar). Residues marked with asterisks do not ablate vanilloid sensitivity when introduced into rVR1.

type receptor, a drop in extracellular pH from 7.6 to 6.3 caused a dramatic potentiation of heat-evoked currents (shown for S512Y, Figure 4E).

Finally, an extended mutational analysis to conserved residues adjacent to S512 (D509, S510, Y511, E513, and I514) (Figure 4B) revealed the most significant effects

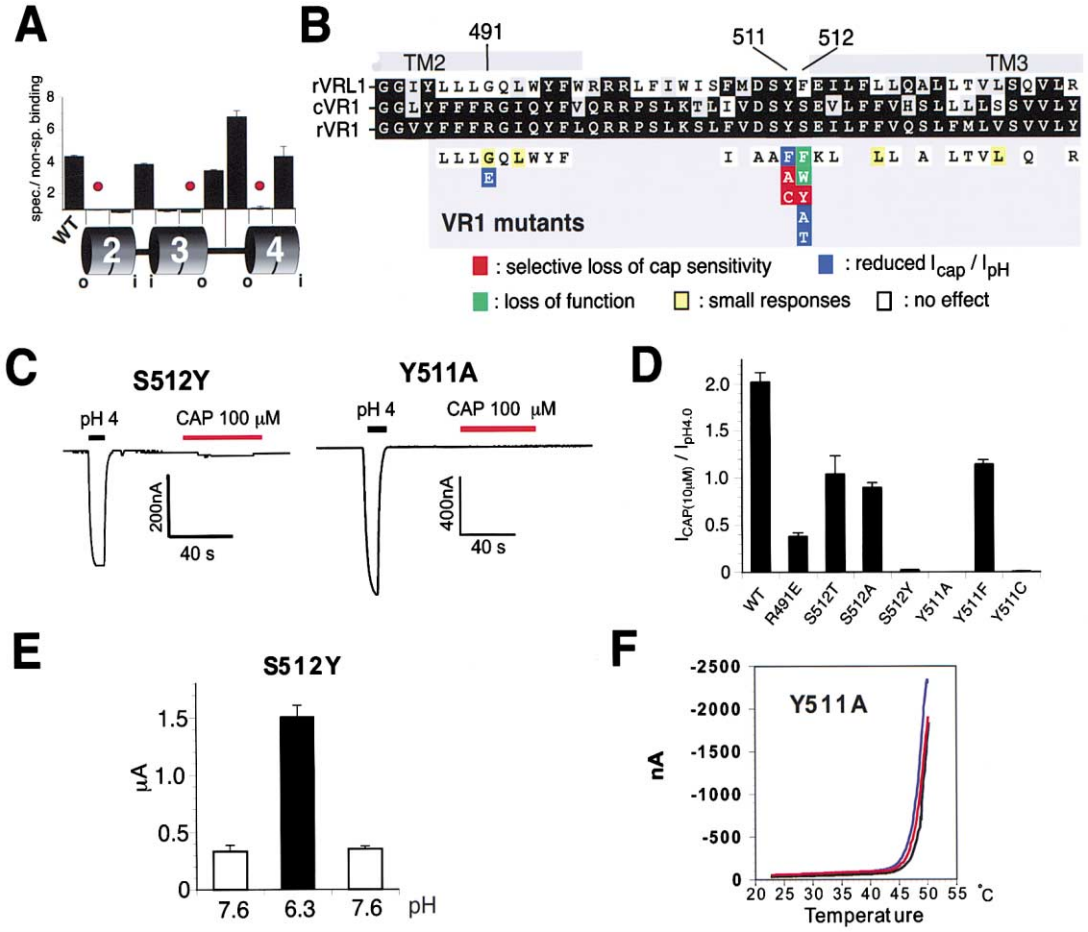

Figure 4. Selective Ablation of Vanilloid Interaction by Mutations within TM2-4 of VR1

(A) ^{3}H-RTX binding was assessed for nine different mini chimeras in which outer (o) or inner (i) transmembrane segments (cylinders) or hydrophilic connecting loops (black lines) of the TM2-4 region of rVR1 were replaced by cognate segments from VRL1. Substitutions within TM 2, 3, or 4 eliminated ligand binding (1.5 μg/ml protein; 500 pM ^{3}H-RTX; n = 2 each). Chimeras TM2-O, TM3-O, and TM4-O (red dots) could be activated by high concentrations of capsaicin (20 μM for 30 s) when expressed in oocytes (amplitudes of 490 ± 60 nA, 270 ± 20 nA, and 1100 ± 200 nA, respectively; n = 3 each).

(B) rVRL1 (top), cVR1 (middle), and rVR1 (bottom) sequences are aligned within the TM2-TM3 region. Conserved residues are shown on black background. Point mutations introduced into rVR1 are shown in the blue box, and those affecting functional properties of the channel are categorized as follows: green indicates loss of function; blue indicates selectively reduced capsaicin sensitivity (versus proton [pH 4]-activated currents); red indicates selective loss of capsaicin sensitivity; yellow indicates reduction in both capsaicin and proton-activated currents; and white indicates mutations with no effect. Essential positions in rVR1 are indicated by numbers.

(C) Representative traces recorded from an oocyte expressing rVR1 mutants S512Y (left) and Y511A (right) demonstrate selective loss of sensitivity to capsaicin (100 μM) versus extracellular protons (pH 4.0).

(D) Quantitative analysis for these and other mutants was carried out by first perfusing cells with an acidic solution (pH 4.0 for 10 s), followed by a 1 min washout, and then capsaicin (10 μM for 30 s). Ratios of the maximal capsaicin-activated and proton-activated currents are shown (n = 3 each, error bars represent SEM).

(E) Heat-evoked responses of the vanilloid-insensitive S512Y mutant are potentiated by extracellular protons (pH 6.3). Protocol as in Figure 1C; peak temperature of heat pulses = 49°C. Averaged absolute peak currents from five experiments are shown.

(F) Heat threshold of current recorded from oocytes expressing rVR1 Y511F mutant channels. Three individual traces are shown. The bath temperature was elevated from room temperature to 50°C within 15 s. Similar to wild-type rVR1, currents activated with a temperature threshold of ~45°C.

with mutant Y511A (Figures 4C and 4D). While lacking any significant capsaicin sensitivity, this channel showed normal heat- and proton-evoked responses with a thermal threshold and current amplitudes that were indistinguishable from those of the wild-type receptor (Figure 4F). Because the aforementioned model suggested that aromatic residues might be important for vanilloid interaction, we tested if the aromatic nature of the residue at position 511 is essential for ligand binding. Indeed, whereas substitution with a phenylalanine had only moderate effects, substitution with a nonaromatic cysteine again eliminated capsaicin sensitivity (Figures 4B and 4D).

Patch-clamp experiments with capsaicin (Tominaga et al., 1998) or a less lipophilic derivative (Jung et al., 1999) suggest that vanilloid agonists cross the plasma membrane to activate VR1 from the intracellular side. According to the current topological model (Vannier et al., 1998; Caterina and Julius, 2001), residues such as

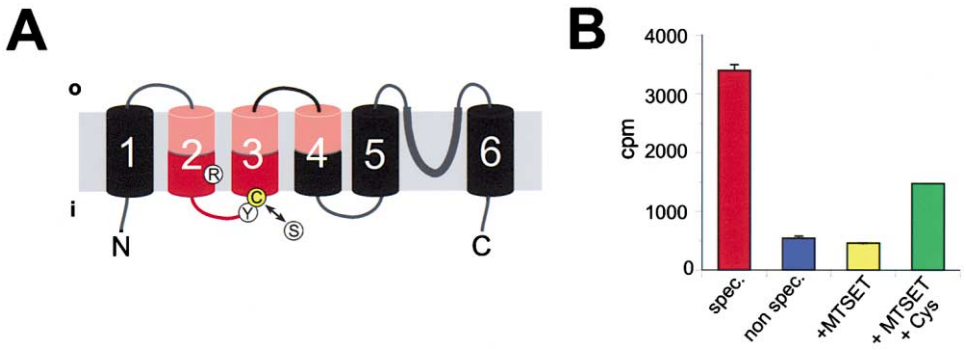

Figure 5. Accessibility of a Critical Residue from the Aqueous Phase

(A) Predicted location of S512, substituted by cysteine in mutant S512C (yellow), is shown within the rVR1 monomer in relation to other residues (R491, Y511) that dramatically reduce vanilloid responses. Light and dark red shading correspond to regions in which substitutions from VRL1 reduce RTX binding or capsaicin-evoked currents, respectively. Outer (o) and inner (i) leaflets of plasma membrane are indicated. (B) The cysteine-reactive reagent MTSET reversibly inhibits ^{3}H-RTX binding to S512C mutant. Untreated membranes from S512C-transfected HEK293t cells showed ~6-fold increase in specific (red) versus nonspecific (blue) ^{3}H-RTX binding. Incubation with MTSET (400 μM) completely eliminated specific binding (yellow), which was partially restored by subsequent reduction with cysteine (green).

Y511 and S512 are located at the transition between an intracellular loop and TM3, where they might interact with the vanilloid ligands at the intracellular face of the membrane (Figure 5A). By using membrane-impermeant-methanethiosulfonate compounds (MTSET and MTSES [Karlin and Akabas, 1998]) to modify a cysteine introduced at position 512, we provide evidence that this position is indeed accessible from the aqueous phase (Figure 5B). ^{3}H-RTX bound to the S512C mutant with a K_D of 310 ± 20 pM (n = 2), only slightly higher than that of the wild-type receptor (200 pM). Binding of ^{3}H-RTX to wild-type VR1 was largely unaffected by incubation with 1 mM MTSET (11% ± 8% reduction; n = 4), but specific binding by the S512C channel was completely eliminated after exposure to 0.4 mM MTSET (Figure 5B). This inhibition could be partially reversed by subsequent reduction with cysteine, demonstrating that covalent modification of S512C does not lead to an irreversible change that precludes vanilloid binding (Figure 5B). Specific binding of ^{3}H-RTX to mutant receptors was also eliminated with another membrane-impermeant cysteine-reactive compound, PEO-maleimide-activated biotin (not shown).

Endogenous Inflammatory Ligands (Endovanilloids) and Vanilloids Rely on the Same Structural Elements for Receptor Activation

Having identified specific structural determinants for vanilloid sensitivity, we asked whether they also play a role in the interaction of VR1 with the putative endogenous agonist (endovanilloid) anandamide (Zygmunt et al., 1999; Piomelli, 2001). Anandamide, a bioactive lipid and structural relative of arachidonic acid, was initially identified as an endogenous ligand of cannabinoid receptors (Devane et al., 1992). Recently, anandamide has been shown to activate vanilloid receptors and displace ^{3}H-RTX binding from VR1-containing membranes, mediating vasodilation and inflammation by activation of capsaicin receptors in sensory nerve terminals (Zygmunt et al., 1999; Gauldie et al., 2001). Anandamide shows significant structural similarity with natural and synthetic vanilloid agonists, such as capsaicin and olvanil, and we therefore asked if the same structural elements that we have identified in our mutational analysis are also important for anandamide interaction. Both cVR1 and rVRL1 were insensitive to anandamide (20 μM) (S.J., H. Chuang, and D.J., unpublished observation). However, anandamide was capable of displacing ^{3}H-RTX from the capsaicin-sensitive VRL1-based chimera V2-4/L (Figure 6A). Moreover, anandamide failed to activate the capsaicin-insensitive Y511A mutant, even under conditions (extracellular pH 6.3) that increase anandamide activity at the wild-type receptor (Figure 6B). Thus, structural determinants of capsaicin sensitivity also appear to be essential for the interaction of VR1 with endogenous agonists.

Inflammatory peptides, such as bradykinin or nerve growth factor (NGF), heighten sensitivity of VR1 to noxious stimuli by binding to their own receptors (BK_2 and trkA, respectively) on sensory neurons and by activating phospholipase C (PLC)-mediated signaling pathways (Julius and Basbaum, 2001). As a result, VR1-mediated currents are observed at normal body temperature, a phenomenon that may contribute to chronic pain in inflammatory settings associated with infection, arthritis, or neuropathy. Indeed, VR1-deficient mice do not develop thermal hyperalgesia in the setting of inflammation (Caterina et al., 2000; Davis et al., 2000) or following treatment with bradykinin or NGF (Chuang et al., 2001). We have recently proposed that potentiation of VR1 is produced, in part, by PLC-mediated hydrolysis of the plasma membrane phospholipid, PIP_2, which exerts an inhibitory influence on channel function (Chuang et al., 2001). Here, we asked whether sensitivity to capsaicin is a prerequisite for potentiation by PLC-coupled receptors. The capsaicin-insensitive VR1 mutant, Y511A, was coexpressed with the PLC-coupled m1 muscarinic acetylcholine receptor in *Xenopus* oocytes (Figure 6C). Mutant channels were first activated by a threshold concentration of protons (pH 5.5), which elicited small inward currents. Subsequent activation of m1 receptors by carbachol produced a dramatic potentiation of these proton-evoked currents (17-fold ± 7-fold, Figure 6C), as

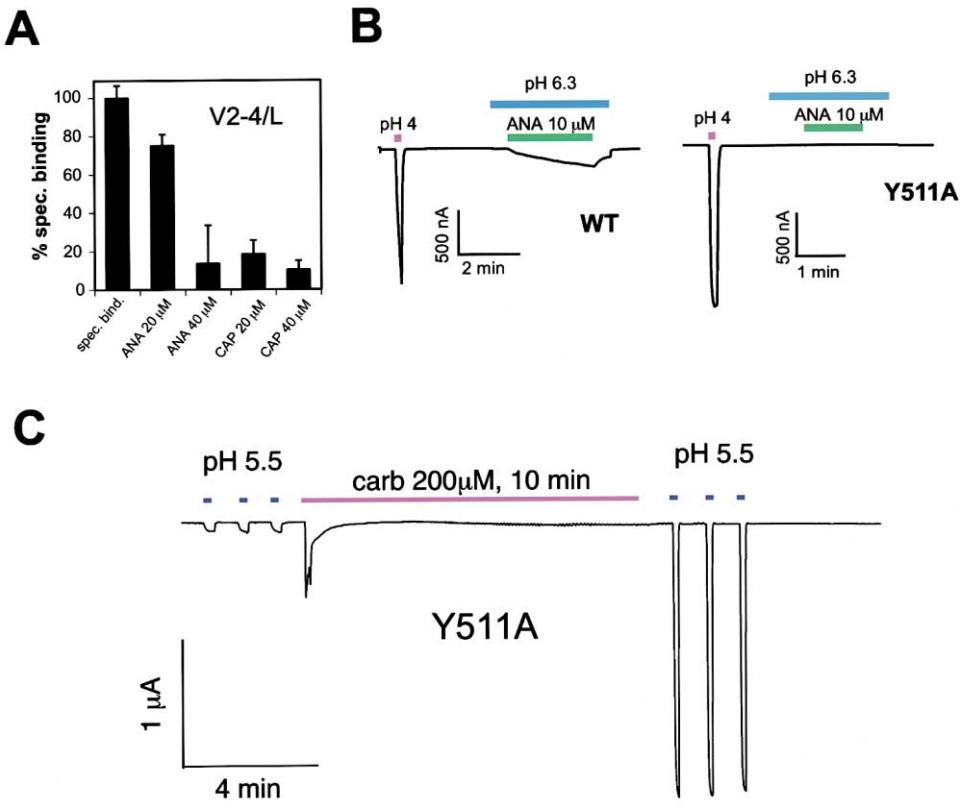

Figure 6. Colocalization of Structural Determinants of Anandamide and Vanilloid Sensitivity, but Not for PIP_2-Mediated Inhibition

(A) Membranes from V2-4/L-transfected HEK293t cells were incubated with 200 pM ^{3}H-RTX in the absence or presence of anandamide or capsaicin at the concentrations shown (n = 4 each). At the highest dose, anandamide reduced specific ^{3}H-RTX binding by >80%, which was comparable to capsaicin.

(B) Representative traces show that wild-type rVR1 responds to extracellular protons (pH 4.0) and to anandamide (10 μM) under mildly acidic (pH 6.3) conditions. In contrast, mutant Y511A was robustly activated by pH 4.0 solution, but failed to respond to anandamide, even under mildly acidic (pH 6.3) conditions.

(C) Potentiation of the capsaicin-insensitive mutant Y511A by activation of m1 muscarinic receptors coexpressed in *Xenopus* oocytes. Y511A channels were activated with three pulses of acidic solution (pH 5.5) for 10 s. Subsequently, the cell was perfused with 200 μM carbachol for 10 min, initially activating calcium-dependent chloride channels. Subsequently, the cell was again perfused with acidic solution, activating strongly potentiated currents (17-fold ± 7-fold; n = 5).

observed with wild-type VR1. In a similar fashion, the capsaicin-insensitive avian receptor ortholog could not be activated by anandamide, but was potentiated by m1 receptor activation (not shown). These results indicate that structures conferring vanilloid sensitivity are not required for PLC-mediated potentiation of VR1.

Discussion

High-affinity cross-linkable vanilloid ligands do not currently exist, and thus sites of contact within the receptor have not been directly identified. Nonetheless, our data, together with pharmacological studies of the native receptor (Szallasi and Blumberg, 1999) and structure of the photosynthetic reaction center (Spyridaki et al., 2000), support a model in which an aromatic residue (e.g., Y511 in rVR1) interacts with the vanillyl-moiety of capsaicin on the cytosolic face of the membrane. Additional polar residues, such as S512 or R491, could interact with capsaicin via hydrogen bonds, whereas lipophilic residues in TM3 may contribute to hydrophobic interactions with the aliphatic moiety of capsaicin within the plane of the membrane. The avian receptor shows residual sensitivity to vanilloid compounds, as evidenced by activation at exceedingly high capsaicin concentrations. We therefore propose that the chicken receptor has a rudimentary vanilloid binding site consisting of highly conserved residues (such as Y511) within or adjacent to TM3. The minimal region of the rat receptor that confers vanilloid sensitivity to the chicken clone contains ~8 mammalian-specific residues that, together with these conserved amino acids, reconstitute a fully functional vanilloid binding pocket in the context of the chick clone.

TRP channels, such as VR1, share structural similarities with K_v-type potassium channels, including the same 6TM topology (Vannier et al., 1998). According to current helix-packing models of K_v channels, derived from helical periodicity analysis (Minor et al., 1999; Hong and Miller, 2000) and crystallographic approaches (Doyle et al., 1998; Clapham, 1999), transmembrane domains S1, S2, and S3 are located on the lipid-facing periphery of the tetrameric channel complex, whereas S5 and S6 are localized to the pore-forming channel

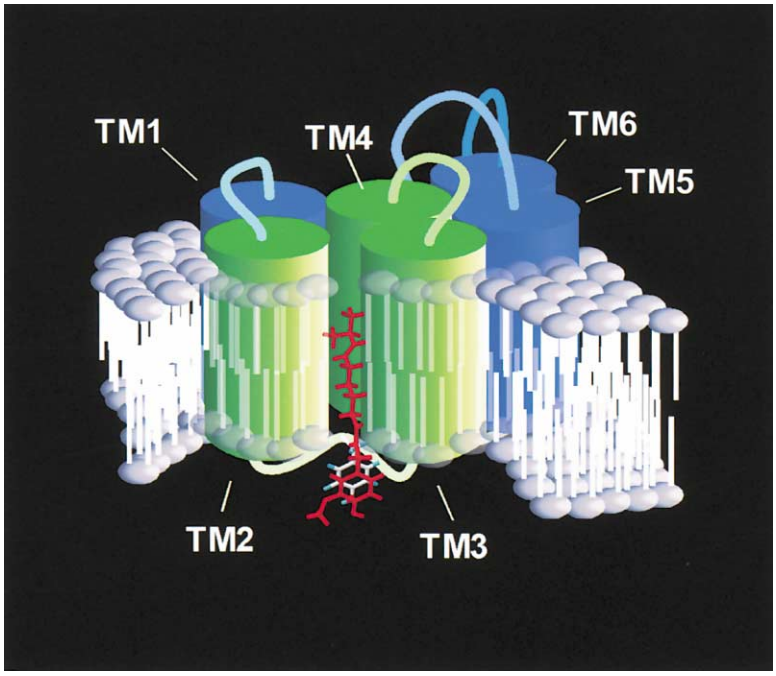

Figure 7. Structural Model of Capsaicin Bound to the Vanilloid Receptor

The transmembrane moiety of the receptor monomer is rendered after a helix-packing model derived from structurally related K_v channels. Similar to voltage-gated potassium channels, VR1 is likely to form a tetramer with the ion-conducting pore in the center, faced by TM6. The vanillyl moiety of capsaicin (red) is shown to interact with an aromatic residue (such as Y511) located in the cytosolic region linking TM2 and TM3. Within the plane of the membrane, the ligand interacts with the TM2-3 region at the channel periphery (green). Structural rearrangements induced by ligand binding may be transduced through TM4 (green) into the channel core. In K_v channels, TM4 (S4) represents the voltage sensor that interacts via salt bridges with TM2 and 3.

core. Assuming similar helix packing for VR1, the lipophilic moiety of capsaicin may bind to transmembrane domains 2 and 3 on the channel-lipid interface, while the vanilloid moiety would interact with residues (such as Y511) in the cytosolic region linking these domains (Figure 7). Ligand-induced structural changes of TM2 and TM3 may affect TM4. In K_v channels, this transmembrane domain (S4) represents the voltage sensor, which is linked via salt bridges to S2 and S3 (Perozo et al., 1994). Voltage-dependent movements of S4 result in opening of the channel pore (Horn, 2000). Similar helix interactions in the capsaicin receptor may finally result in an allosterically induced facilitation of channel activation by heat or protons.

The sequences of the avian capsaicin receptor and its mammalian counterparts show an unusually high divergence (only 68% amino acid identity), compared to a much higher degree of conservation for receptors and channels in the central nervous system (e.g., >95% for $GABA_A$ receptors). Indeed, other sensory receptors, such as those in the olfactory or taste systems (Adler et al., 2000; Nelson et al., 2001), also show relatively low degrees of sequence conservation among species. This phenomenon suggests that receptors for physical or chemical sensory stimuli tolerate a higher degree of variation, or are subject to strong evolutionary pressure, enabling species to establish their ecological niches. Indeed, our findings suggest that structural differences between avian and mammalian VR1 orthologs have important consequences for both plants and animals. For the pepper plant, these differences appear to underlie the phenomenon of directed deterrence, whereby mammalian predators are repelled, whereas birds are favored as vectors for seed dispersal (Tewksbury et al., 1999; Tewksbury and Nabhan, 2001).

Experimental studies in diverse systems, such as the *Drosophila* phototransduction pathway (Scott and Zuker, 1998; Hardie and Raghu, 2001), mammalian hippocampal neurons (Strübing et al., 2001), and sensory neurons (Chuang et al., 2001; Tominaga et al., 2001) show that the regulation of TRP-like channels by PLC-coupled receptors may represent a very ancient and ubiquitous regulatory pathway (Hofmann et al., 2000; Clapham et al., 2001). Indeed, our results show that both avian and mammalian vanilloid receptors are potentiated downstream of PLC-coupled receptors. Thus birds, like mammals, may use a similar regulatory mechanism to generate thermal hyperalgesia in response to proinflammatory and proalgesic peptides, nucleotides, or neurotrophic factors (Gentle and Hunter, 1993; Koltzenburg and Lewin, 1997; Chuang et al., 2001; Tominaga et al., 2001) that promote PIP_2 hydrolysis. In contrast, we found that mammalian, but not avian channels can detect endogenous vanilloid-like agonists, such as anandamide. The reported insensitivity of reptiles and toads to capsaicin (Hawkins et al., 1991; Szallasi and Blumberg, 1999) further suggests that mammalian vanilloid receptors evolved from an ancestral channel capable of integrating acidity and noxious heat. Thus, the same structural elements that help define the chili pepper's ecological niche may also determine the extent to which endogenous lipids contribute to inflammatory thermal hyperalgesia in a given vertebrate species. This newly acquired sensitivity to anandamide (and perhaps other, structurally related lipids) may provide selective pressure to maintain vanilloid sensitivity among mammals.

Experimental Procedures

Molecular Biology

cVR1 was cloned by screening two independent chicken DRG cDNA libraries under low stringency conditions with two ^{32}P-labeled probes generated from rVR1 cDNA restriction fragments. Of more than 50 positive clones, 21 contained inserts of >2 kb in size. Of

these, 15 were inserted into pcDNA3 (Invitrogen) and sequenced. These clones contained overlapping partial sequences or full-length open reading frames of the chicken VR1 ortholog, cVR1, coding for a protein with a length of 843 amino acids (GenBank accession number AY072909). For oocyte expression, cDNAs were inserted into the pGEM-HE oocyte expression vector. We also identified a second cDNA with homology closer to mammalian VRL1 (not shown). No other homologous cDNAs were identified in these screens. Chimeras of VR1 and VRL1 were generated using random bacterial recombination (Moore and Blakely, 1994) and analyzed using oligonucleotide hybridization followed by sequencing. Chimeric elements were later combined by restriction cloning or by recombinant PCR. Point mutations were introduced using oligonucleotide-directed mutagenesis as previously described (Jordt et al., 2000). All constructs were verified by DNA sequencing. cDNAs were cloned into pCDNA3 or the combined oocyte/mammalian expression vector pFROG3 containing 5′- and 3′- untranslated regions of *Xenopus* β-globin (Jordt et al., 2000). Rat OTRPC4 cDNA was amplified by 5′- and 3′-RACE PCR from rat kidney Marathon cDNA (Clontech) using degenerate oligonucleotides based on mouse ESTs W53556 and AI510567. The encoded rat protein was 98% identical to the mouse OTRPC4 sequence (Strotmann et al., 2000) and matched that of the rat sequence (Liedtke et al., 2000). In our sequence, as in all published OTRPC4 sequences, no stop codon in the 5′-UTR could be identified to unequivocally assign an initiation codon.

Binding Assays

Membrane preparation and ^{3}H-RTX binding experiments were performed as described (Szallasi et al., 1999). For all experiments, a ≥15-fold excess of free radioactive ligand was used. All incubations were performed at 37°C. Nonspecific binding was determined in the presence of 1000-fold excess of nonradioactive ligand. For SCAM-analysis, rVR S512C-containing membranes (10 μg protein) were incubated for 20 min with 400 μM MTSET in 1 ml reaction buffer (Javitch et al., 1995). Half of the reaction mix was immediately diluted in 20 ml binding buffer, the other half after incubation with 5 mM cysteine for 10 min. Aliquots containing 1 μg protein were tested for ^{3}H-RTX binding (300 pM).

Electrophysiology

Templates were linearized and transcribed with T7 polymerase (AmpliScribe, Epicentre). Oocytes were injected with 5–10 ng cRNA. Two electrode voltage clamp analysis ($E_h = -40$ mV) was carried out 4 to 9 days postinjection. Recordings were performed as previously described (Jordt et al., 2000). All procedures involving the care and use of frogs were carried out in accordance with federal and UCSF guidelines.

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