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Agonist Recognition Sites in the Cytosolic Tails of Vanilloid Receptor 1*

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Vanilloid receptor 1 (VR1), a ligand-gated ion channel activated by vanilloids, acid, and heat, is a molecular detector that integrates multiple modes of pain. Although the function and the biophysical properties of the channel are now known, the regions of VR1 that recognize ligands are largely unknown. By the stepwise deletion of VR1 and by chimera construction using its capsaicin-insensitive homologue, VRL1, we localized key amino acids, Arg-114 and Glu-761, in the N- and C-cytosolic tails, respectively, that determine ligand binding. Point mutations of the two key residues resulted in a loss of sensitivity to capsaicin and a concomitant loss of specific binding to [^{3}H]resiniferatoxin, a potent vanilloid. Furthermore, changes in the charges of the two amino acids blocked capsaicin-sensitive currents and ligand binding without affecting current responses to heat. Thus, these two regions in the cytoplasmic tails of VR1 provide structural elements for its hydrophilic interaction with vanilloids and might constitute a long-suspected binding pocket.

Capsaicin, the principal pungent ingredient of hot peppers, excites sensory neurons by opening an ion channel, the vanilloid receptor 1 (VR1),[1] thereby causing pain. VR1 is a ligand-gated, cationic channel that is present mainly in small nociceptive sensory neurons (1–3). The presence of VR1 in sensory neurons leads to questions concerning the existence of endogenous capsaicin-like substances, and various lipid metabolic products of lipoxygenases or anandamide have been suggested as candidates, because they activate VR1 and are structurally similar to capsaicin (4, 5). Accordingly, a role for lipoxygenase products in the activation of VR1 during inflammation was suggested (5), and in fact, bradykinin, a potent pain-causing inflammatory mediator, is now known to activate VR1 via the lipoxygenase/VR1 pathway (6). In addition, bradykinin also has a potential to sensitize VR1 via a phospholipase C or protein kinase C pathway (7–9).

VR1 is also activated by acid or heat at over 43 °C, a threshold temperature for pain (3, 10–12). Moreover, because ischemic or inflamed tissues become acidic, the acid activation of VR1 is a pathologically relevant event (13). More direct evidence for the pathophysiological role of VR1 in the production of inflammatory pain came from knock-out experiments. In mice lacking VR1, thermal hyperalgesia evoked by inflammation is reduced (14, 15). Furthermore, hyperalgesia induced by the key inflammatory mediators, bradykinin and nerve growth factor, is reduced in mice lacking VR1 (8). Thus, VR1 is now considered a primary molecular transducer that mediates inflammatory hyperalgesia (13).

The putative topology of VR1 indicates that it belongs to a class of transient receptor potential channels possessing six transmembrane domains and two cytosolic domains at each N- and C terminus (3, 16). VR1 appears to form a homotetramer when expressed heterologously (17). However, VR1 may form a heteromultimer with another temperature-sensitive channel, transient receptor potential V3 (18). The cloning of VR1 resulted in the identification of many genes with sequence homology, for example, heat-sensitive vanilloid receptor-like (VRL1) channel and osmotically activated channel (VR-OAC) (19, 20). In addition, splicing variants of VR1, such as stretch-inhibitable channel (SIC) and VR.5′sv, were also identified (21, 22). Interestingly, none of these homologues respond to capsaicin when expressed heterologously.

Capsaicin, *N*-(4-hydroxy-3-methoxybenzyl)-8-methylnon-6-enamide, is known to have three functional regions, *i.e.* a homovanillic acid, an amide bond, and an aliphatic chain, which are often referred to as the A, B, and C regions, respectively (23). The homovanillyl motif and amide bond regions contain dipolar groups, such as hydroxyl and carbonyl groups, which are implicated in hydrogen bonding interactions. Analyses of the structure-activity relationship of numerous capsaicin analogues suggest that these polar regions in capsaicin analogs are essential for maintaining pungency, which could be alternatively expressed as maintaining the excitation of sensory neurons (23–26). In contrast, the aliphatic chain in the C region, which has an optimal chain length of 8–10 carbon atoms, is presumed to interact hydrophobically with its receptors. Although a receptor model has been suggested based on the structure-activity relationships of the capsaicin analogs, structural elements in VR1 that confer specific interactions with vanilloids have not been characterized. Recently, a region spanning the third transmembrane domain (TM3) in VR1 was found to be essential for ligand binding, presumably by hydrophobic interaction with capsaicin (27). However, regions in VR1 that determine its binding to ligands, with an emphasis on hydrophilic interactions, have not been resolved. Moreover, unlike other ligand-gated channels that produce fast synaptic transmission, vanilloids are known to act on the capsaicin receptor from the intracellular side (27–29). This suggests that certain regions in the cytosolic tails of VR1 are targeted as

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[1] The abbreviations used are: VR1, vanilloid receptor 1; TM, transmembrane; HEK, human embryonic kidney; RTX, resiniferatoxin; PBS, phosphate-buffered saline; VRL1, vanilloid receptor-like channel 1.

ligand recognition sites. Because of the innate implication of the involvement of cytosolic tails in ligand binding, we constructed VR1/VRL1 chimeras and performed a series of deletions and site-directed mutagenesis at the N and C termini of VR1 to localize amino acid residues in each cytosolic tail that appeared critical for ligand binding.

EXPERIMENTAL PROCEDURES

Molecular Biology—A cDNA clone encoding an open reading frame of VR1 was isolated from the dorsal root ganglion neurons of adult rats, as described previously (28). A VRL1 cDNA was a generous gift from D. Julius of the University of California, San Francisco. The cDNA of wild-type VR1 and its various mutants were inserted into pSDTF (a generous gift from T. P. Snutch of British Columbia University) and pCDNA3 (Invitrogen) to obtain expression in oocytes and human embryonic kidney (HEK) 293T cells, respectively. VR1/VRL1 chimeras were constructed by recombinant PCR. The deletion mutants of VR1 from either N- or C-terminal cytoplasmic domains were produced by PCR. Site-directed mutagenesis was performed using a combination of two overlapping PCR fragments, which were constructed from mutagenic primers (30). The incorporation of engineered mutations and the fidelity of the PCR were confirmed by DNA sequencing. cDNAs were linearized with *Xba*I. Capped mRNAs were synthesized using an SP6 RNA polymerase in an Ambion Megascript kit, as described by the manufacturer. Expressions in oocytes of all mutants of VR1 or VR1/VRL1 chimeras were confirmed by immunoblots.

Electrophysiology—VR1 and its mutants were expressed in *Xenopus* oocytes as described previously (28). Briefly, stage V-VI oocytes from *Xenopus laevis* were defolliculated with type A collagenase (Roche Molecular Biochemicals) and microinjected with 5–25 ng of the cRNAs of wild-type VR1, mutant VR1s, or VR1/VRL1 chimeras in 50 nl of diethylene pyrocarbonate-treated water. 2 to 4 days after the microinjection, capsaicin-sensitive currents were recorded using the two-electrode voltage-clamp technique. The membrane potential was held at −60 mV, and the recording solution contained the following (in mM): 96 NaCl, 5 HEPES, 2 KCl, 1.8 $CaCl_2$, and 1 $MgCl_2$ (pH 7.5). All experiments were performed at room temperature.

[^{3}H]RTX Binding Assay—Wild-type VR1 and its mutant cDNAs subcloned in pCDNA3 were transfected into HEK 293T cells using a LipofectAMINE PLUS kit (Invitrogen). 24 to 48 h after transfection, transfected cells were washed with phosphate-buffered saline (PBS), resuspended in PBS containing 5 mM EDTA and stored at −70 °C until assayed. The binding assay was carried out in 96-well filtration plates (31) (Multiscreen separation system; Millipore) fitted with polyvinylidene difluoride membrane filters (pore size = 0.45 μm; Durapore). 1×10^5 cells were then loaded into wells that had been washed with assay buffer containing the following (in mM): 5 KCl, 5.8 NaCl, 0.75 $CaCl_2$, 2 $MgCl_2$, 320 sucrose, 10 HEPES (pH 7.4) containing 0.25 mg/ml bovine serum albumin (Cohn fraction V; Sigma). Cells were incubated with [^{3}H]RTX (30–1,000 pM) in a total reaction volume of 150 μl for 1 h, with gentle shaking at 37 °C. Nonspecific binding was defined as [^{3}H]RTX binding in the presence of 1 μM non-radioactive RTX (32, 33). After 1 h, the microplate was chilled on ice, and prechilled α_1-acid glycoprotein (1 mg/well; Sigma) in 50 μl of assay buffer was added to each well to reduce nonspecific binding (33, 34). The solution in the microplates was then aspirated immediately, and the microplate filter membranes were washed with a buffer containing 100 μg of α_1-acid glycoprotein, dried completely, and collected for liquid scintillation counting.

The binding data were calculated using the Hill equation, $B = (B_{max} \times L_H^n)/(K_D^n + L_H^n)$, where B represents the concentration of receptor-ligand complex, B_{max} is the maximal binding capacity, L_H is the concentration of radioactive free ligand, K_D is the concentration of [^{3}H]RTX when half of the receptors are occupied, and n is the Hill coefficient.

Immunoblot—Lysates of the oocytes injected with the cRNAs of the wild-type or mutant VR1s were obtained by centrifuging at $1,000 \times g$ for 10 min after homogenizing the cells in PBS containing 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. Equal amounts of whole cell lysates were separated by 12% SDS-PAGE after incubation for 10 min at 55 °C. Electroblotted membranes were incubated with antiserum raised against the N-terminal cytoplasmic domain of VR1 (NVR1) or with antiserum against the C-terminal cytoplasmic domain of VR1 (CVR1), as described previously (35). Peroxidase-conjugated goat anti-mouse IgG and an enhanced chemiluminescence detection kit (Amersham Biosciences) were used for the visualization.

Immunocytochemistry—HEK 293T cells transfected with VR1 and its mutants were seeded on a cover glass coated with poly-L-lysine. After incubation for 24 h, cells were washed briefly three times with ice-cold PBS containing 0.05% bovine serum albumin and fixed for 10 min in PBS containing 10% formaldehyde at room temperature. Cells were then washed three times with PBS, permeabilized with PBS containing 0.1% (v/v) Triton X-100 for 5 min at room temperature, and rewashed. The cells were then rinsed with 2% bovine serum albumin for 1 h, to block the nonspecific binding of proteins other than antibodies, and incubated with polyclonal anti-NVR1 or -CVR1 antiserum (1:1000 dilution) at 4 °C overnight. After washing, the cells were incubated with fluorescein isothiocyanate-conjugated rabbit antimouse IgG (1:100 dilution; Zymed Laboratories Inc.) for 1 h, rewashed extensively, and incubated with propidium iodide (Sigma) for nuclear staining. Cells expressing VR1 and its mutants were viewed under a confocal laser scanning microscope (Leica TCS; Wetzlar, Germany).

Materials—[^{3}H]RTX (48.0 Ci/mmol) was purchased from Amersham Biosciences, non-radioactive RTX was from Biomol Research Laboratories, Inc., and capsaicin and capsazepine were from Research Biochemicals, Inc. (Natick, MA). Reagents used in cell culture were purchased from Invitrogen, and all the other reagents were from Sigma, unless indicated otherwise.

Statistics—Data are presented as means ± S.E. Multiple comparisons of means were performed using one-way analysis of variance followed by Tukey's *post hoc* test. The Student's t test was used to compare two means. p values smaller than 0.05 were considered significant.

RESULTS

VR1/VRL1 Chimeras—The issue of the requirement of cytosolic tails for ligand binding was addressed by constructing a series of chimeras between VR1 and a capsaicin-insensitive VR1 homologue, VRL1. When 1 μM capsaicin was applied to *Xenopus* oocytes, those expressing wild-type VR1 exhibited large inward currents (I_{cap}) at a holding potential of −60 mV, which were blocked by 10 μM capsazepine, a competitive antagonist of the capsaicin receptor (2, 36) (Fig. 1, *A* and *B*). VRL1 expressed in oocytes did not respond to capsaicin. Chimeras (chimeras *c* and *d*) constructed with the VRL1 backbone but containing either the N or C terminus of VR1, failed to exhibit I_{cap}, suggesting the possible role of the two cytosolic regions in capsaicin activation. When both cytosolic tails of VR1 were substituted for the corresponding segments of VRL1, the chimera (chimera *e*) still did not respond to capsaicin. The lack of activation by capsaicin in chimera *e* could have been because of the lack of the VR1 functional TM3, which is implicated in hydrophobic interaction with vanilloids (27). Indeed, when the TM3 region of VR1 was added, the VR1/VRL1 chimeras (chimeras *f* and *g*) elicited I_{cap}. In contrast, when either TM3 or the two cytosolic tails of VR1 were replaced by cognate segments of VRL1, the chimeras produced (chimeras *h*, *i*, and *j*) failed to show I_{cap}, even in the presence of maximal concentration (20 μM) of capsaicin. These results indicate that certain regions in the N and C termini of VR1 are required, in addition to the TM3 regions for VR1 activation by capsaicin.

Serial Deletions at the N terminus of VR1—To localize regions in the cytosolic tails that confer capsaicin sensitivity, a series of deletion mutants of the N terminus of VR1 were constructed (Fig. 2*A*). When 1 μM capsaicin was applied, oocytes expressing VR1 mutants deleted up to Asp-113, such as Δ^{1-35}, Δ^{1-109}, and Δ^{1-113}, produced I_{cap} comparable with that of wild-type VR1 (Fig. 2, *B* and *C*). In contrast, current responses were completely absent in oocytes expressing a series of VR1 deletion mutants that omitted Arg-114, namely Δ^{1-114}, Δ^{1-115}, Δ^{1-116}, Δ^{1-117}, Δ^{1-118}, Δ^{1-119}, Δ^{1-151}, Δ^{1-199}, and Δ^{1-432}. Moreover, the insensitivity to capsaicin of the VR1 deletion mutants lacking Arg-114 was not because of the lack of mutant expression in oocytes. All mutants were found to be expressed properly to the same extent as wild-type VR1 when probed by immunoblots with a mouse antiserum raised against a segment of the C terminus, VR1 (684–838) (Fig. 2*D*). When transfected to HEK 293T cells, the Δ^{1-114} mutant showed immunoreactiv-

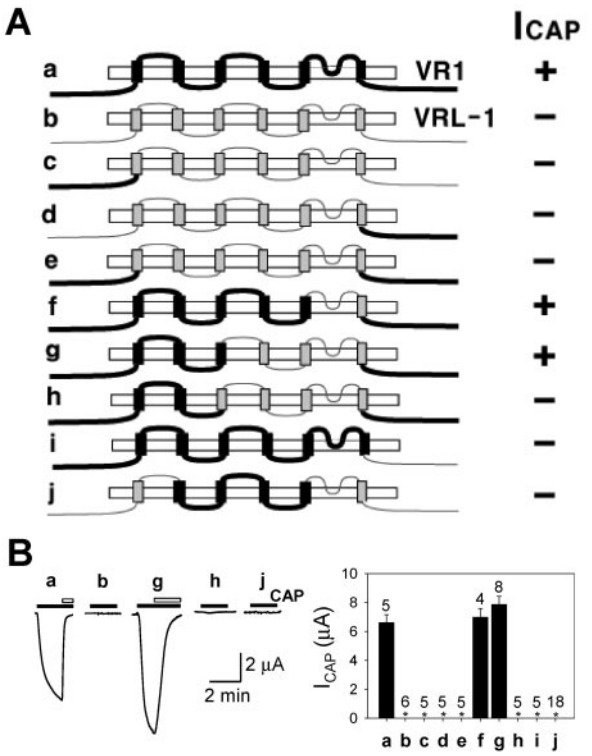

FIG. 1. **Chimera construction from VR1 and VRL1 suggests that both cytosolic tail and transmembrane domain 3 regions are necessary for the activation of VR1 by vanilloids.** *A*, chimeras constructed from VR1 and VRL1 and their responses to 1 μM capsaicin when expressed in oocytes. + and − indicate the presence and the absence of current response to capsaicin (I_{cap}), respectively. *B*, representative tracings of VR1/VRL1 chimeras, constructed as shown in *A*, in response to 1 μM capsaicin (*filled bar*). The *open bar* represents the application of 10 μM capsazepine. *Right panel*, summary of I_{cap} of the VR1/VRL1 chimeras. *Numbers above* the *bar* indicate the number of experiments. *, $p < 0.001$ compared with I_{cap} of wild-type VR1.

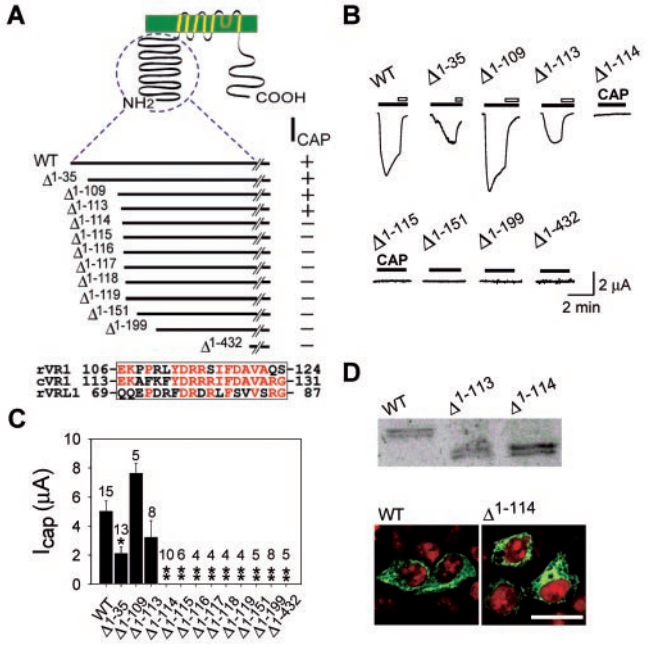

FIG. 2. **Arg-114 and its vicinity in the N terminus of VR1 are required for current responses to capsaicin.** *A*, deletion mutants of VR1 at the N-terminal cytoplasmic tail. The *numbers* on the *left* of the *horizontal bar* indicate deleted VR1 segments. *WT* represents VR1 wild-type. *Lower panel*, sequence alignment in the vicinity of the putative binding site of rat (*rVR1*), chick VR1 (*cVR1*), and rat VRL1 (*rVRL1*). Conserved amino acid residues are indicated in *red*. *B*, example traces of current responses to 1 μM capsaicin (*filled bar*) by *Xenopus* oocytes injected with the cRNAs of VR1 or of its deletion mutants. The *open bar* represents the application of 10 μM capsazepine. *C*, summary of I_{cap} of VR1 and its deletion mutants. *Numbers above* the *bar* indicate the number of experiments. *, $p < 0.01$; **, $p < 0.001$. *D*, immunoblots and immunostaining of VR1 and its mutants. *Upper panel*, cell lysates of *Xenopus* oocytes injected with mutant cRNAs were immunoblotted with mouse antiserum raised against the C-terminal cytoplasmic domain of VR1 (*CVR1*; see text for details). *Lower panel*, HEK cells transfected with wild-type VR1 or Δ^{1-114} mutant were immunostained with fluorescein isothiocyanate-labeled anti-CVR1 (*green*). The nucleus was also stained with propidium iodide (*red*). *Scale bar* represents 20 μm.

ity in the cell membrane and in the cytosol, an expression pattern similar to that of the wild-type VR1 (Fig. 2*D*), and which, in part, indicates membrane targeting. These results indicate that the region close to Arg-114 is essential for channel activation by capsaicin.

Serial Deletions at the C terminus of VR1—Although Arg-114 and the vicinity of Arg-114 in the N terminus appear to be critical for activating VR1, studies on VR1/VRL1 chimeras (particularly chimera *i* in Fig. 1) indicate that a certain region in the C terminus of VR1 is also implicated in activation by vanilloids. Thus, we constructed a series of deletion mutants that lacked portions of the peptide sequence in the C terminus (Fig. 3*A*). When expressed in oocytes, deletion mutants up to Asp-765, such as $\Delta^{823-838}$, $\Delta^{787-838}$, $\Delta^{766-838}$, and $\Delta^{765-838}$, elicited an I_{cap} that was comparable with that of the wild-type VR1 (Fig. 3, *B* and *C*). When deletion was proceeded further to the vicinity of Glu-761 to produce VR1 mutants such as $\Delta^{764-838}$, $\Delta^{763-838}$, and $\Delta^{762-838}$, I_{cap} was reduced significantly compared with that of the wild-type. When deletion proceeded beyond Glu-761, to produce $\Delta^{761-838}$, $\Delta^{732-838}$, and $\Delta^{684-838}$, I_{cap} was undetectable (Fig. 3, *B* and C). Moreover, mutants $\Delta^{761-838}$ and $\Delta^{762-838}$ were found to be expressed properly at ~85 kDa, at levels comparable with that of the wild-type when immunostained with polyclonal antibody raised against a protein segment in the N terminus, VR1 (1–432) (Fig. 3*D*). Moreover, HEK 293T cells transfected with the $\Delta^{761-838}$ mutant showed immunoreactivity in the cell membrane and in the cytosol, which was similar to that of wild-type VR1 (Fig. 3*D*). These results suggest that Glu-761 and its vicinity in the C terminus are necessary for the activation of VR1 by capsaicin.

Binding Ability of VR1 and of VR1 Mutants for [^{3}H]Resiniferatoxin—The ability of deletion mutants to bind [^{3}H]RTX, a potent agonist of the capsaicin receptor, was examined (32). As shown in Fig. 4*A*, [^{3}H]RTX displayed a saturable binding to HEK-VR1 cells. As reported previously (33), half-maximal binding (K_D) occurred at 98.0 ± 3.5 pM [^{3}H]RTX (mean ± S.E., triple determinations, $n = 3$). At K_D, nonspecific binding reached ~12% of the total binding. The Hill coefficient (n) was 1.6 ± 0.04, indicating a positive cooperativity (33). Maximal specific binding (B_{max}) was 211.2 ± 8.4 fmol/10^6 cells.

An N terminus deletion mutant, Δ^{1-109}, that elicited I_{cap} retained specific binding for [^{3}H]RTX with a K_D of 258.1 ± 8.3 pM (mean ± range, triple determinations). In contrast, cells expressing Δ^{1-114}, which failed to show I_{cap}, completely lost specific binding to [^{3}H]RTX (triple determinations, $n = 2$; see Fig. 4*B*). Similarly, deletion mutants at the C terminus, such as $\Delta^{762-838}$ and $\Delta^{763-838}$, which displayed smaller I_{cap}, bound [^{3}H]RTX with K_D values of 382.7 ± 11.2 pM (triple preparations, $n = 3$) and 115.7 ± 43.0 pM (triple determinations, $n = 4$), respectively. In contrast, $\Delta^{761-838}$, which failed to elicit I_{cap}, had no ability to specifically bind [^{3}H]RTX (triple determinations, $n = 2$; see Fig. 4*C*). Thus, deletion mutants that lacked one of these two regions lost current sensitivity to capsaicin and the ability to bind ligands. These results suggest that the

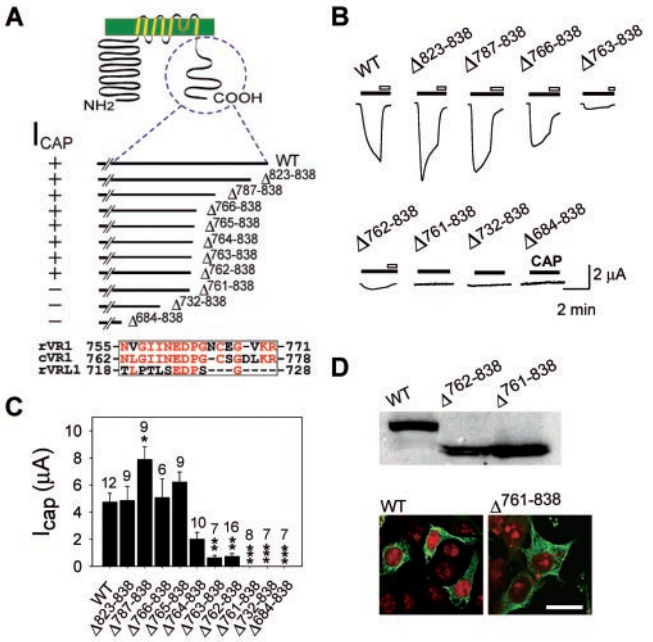

FIG. 3. **Glu-761 and its vicinity in the C terminus of VR1 are also necessary for I_{cap}.** *A*, deletion mutants of VR1 at the C-terminal cytoplasmic tail. The *numbers* on the *right* of the *horizontal bar* indicate deleted VR1 segments. *Lower panel*, sequence alignment of rat (*rVR1*), chick VR1 (*cVR1*), and rat VRL1 (*rVRL1*). Conserved amino acid residues are indicated in *red*. *B*, example traces of current responses to 1 μM capsaicin (*filled bar*) by *Xenopus* oocytes injected with the cRNAs of VR1 or of its deletion mutants. The *open bar* represents the application of 10 μM capsazepine. *C*, summary of I_{cap} of VR1 and its deletion mutants. *Numbers above* the *bar* indicate the number of experiments. *, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.0001$. *D*, immunoblots and immunostaining of VR1 and its mutants. *Upper panel*, cell lysates of *Xenopus* oocytes injected with mutant cRNAs were immunoblotted with mouse antiserum raised against the N-terminal cytoplasmic domain of VR1 (*NVR1*; see text for details). *Lower panel*. HEK cells transfected with wild-type VR1 or $\Delta^{761-838}$ mutant were immunostained with fluorescein isothiocyanate-labeled anti-NVR1 (*green*). The nucleus was also stained with propidium iodide (*red*). *Scale bar* represents 20 μm.

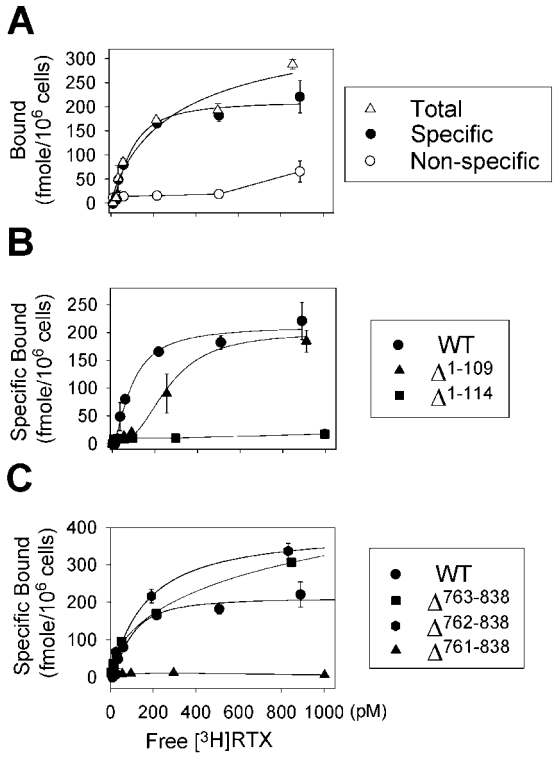

FIG. 4. **VR1 mutants lose specific binding to [^{3}H]resiniferatoxin ([^{3}H]RTX), a potent agonist of VR1.** *A*, wild-type VR1 transfected into HEK cells displayed a saturable specific binding to [^{3}H]RTX. Each data point was fitted to the Hill equation (see "Experimental Procedures" for details). Half-maximal binding (K_D) occurred at 98.0 ± 3.5 pM [^{3}H]RTX (mean ± S.E., triple determinations, $n = 3$). At the K_D, the nonspecific binding reached ~12% of the total binding. *B*, specific binding of wild-type VR1 (*WT*) and of the N-terminal deletion mutants to [^{3}H]RTX. Note that the Δ^{1-109} mutant elicits specific binding (triple determinations) comparable with that of the wild-type VR1 but that Δ^{1-114} fails to exhibit binding to [^{3}H]RTX ($n = 2$, triple determinations). *C*, specific binding of wild-type VR1 and of the C-terminal deletion mutants to [^{3}H]RTX. Note that the deletion mutant, $\Delta^{761-838}$, which shows no capsaicin-sensitive current, fails to elicit specific binding to [^{3}H]RTX ($n = 2$, triple determinations).

two regions in the vicinity of Arg-114 and Glu-761 are critical for ligand binding and that the loss of these regions abrogates vanilloid activation.

Point Mutation at Arg-114 or Glu-761—To determine further whether the two residues, Arg-114 and Glu-761, are required to bind vanilloids, we constructed VR1 mutants that lacked only the single amino acids *i.e.* Arg-114 (Δ^{114}) or Glu-761 (Δ^{761}) (Fig. 5*A*). Our results showed that a deletion as small as a single amino acid affects the ligand binding. As shown in Fig. 5*B*, Δ^{114} and Δ^{761} failed to elicit capsaicin-sensitive inward currents. Furthermore, the mutants did not show specific binding to [^{3}H]RTX (Fig. 5*C*). Because VR1 is activated by acid and heat (3, 12), these mutants were also tested for acid and heat sensitivity in transfected HEK cells. As shown in Fig. 5*D*, both mutants elicited whole-cell currents when activated by heat at ~46 °C, but both mutants failed to respond to acid (pH 5.5). HEK cells transfected with the wild-type VR1 elicited a sustained current response to acid (pH 5.5) and rapid activating and inactivating currents. Moreover, HEK cells transfected with Δ^{114} and Δ^{761} also elicited rapid activating and inactivating currents, which are presumably mediated by native acid-sensing ion channels in HEK cells, because similar acid-sensitive currents were observed in non-transfected HEK cells (37). These results further confirm that the Arg-114 and the Glu-761 regions are critical for ligand binding.

Changes in the Charges of Arg-114 and Glu-761—Because the Arg-114 and Glu-761 residues are charged, it is likely that these charges are necessary for vanilloid binding. We thus constructed VR1 mutants with opposite charges but similar side chain lengths at Arg-114 or Glu-761. As shown in Fig. 6*A*, when positively charged arginine at 114 was replaced by a neutral amino acid, alanine, the mutant (R114A) elicited an I_{cap} comparable with that of the wild-type VR1. However, when the arginine at 114 was replaced by negatively charged glutamate (R114E), a significant reduction (96.9 ± 0.5% reduction, $p < 0.0001$, $n = 13$) in I_{cap} was observed with no apparent specific [^{3}H]RTX binding (Fig. 6, *A* and *B*). Because the adjacent amino acid, Arg-115, is also positively charged, we replaced it with aspartate (R115D). The R115D mutant also abolished the capsaicin-sensitive currents ($p < 0.001$, $n = 6$), indicating that charge at Arg-115 contributes equally to activation by capsaicin (data not shown).

Similarly, when the negatively charged glutamate at 761 was changed to glutamine, a neutral amino acid that retains a similar structure, the mutant (E761Q) elicited a great reduction in I_{cap} (98.0 ± 0.3% reduction, $p < 0.0001$, $n = 10$) and had no specific binding for [^{3}H]RTX (Fig. 6, *A* and *B*). Furthermore, when the glutamate at 761 was substituted with positively charged lysine, the mutant (E761K) showed no current response to capsaicin and ability to bind [^{3}H]RTX (Fig. 6, *A* and *B*). R114E and E761K elicited current responses to heat but not to acid (pH 5.5) (Fig. 6*C*). These results indicate that the negative charge of glutamate at 761 is necessary for ligand

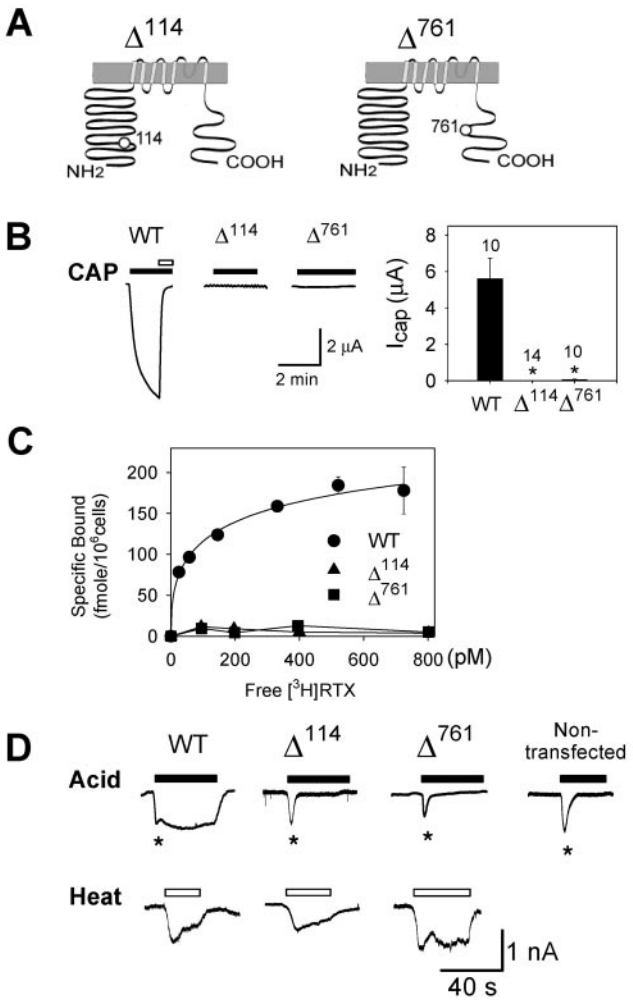

FIG. 5. **Δ^{114} and Δ^{761} mutants of VR1, lacking either of the two putative amino acids in each terminus, Arg-114 and Glu-761, show no I_{cap} and no specific binding to [^{3}H]RTX.** *A*, membrane topologies of the Δ^{114} and Δ^{761} mutants. *B*, current responses to 1 μM capsaicin of wild-type VR1 and of the two mutants in oocytes. *Right panel*, summary of I_{cap} of VR1 and of the two mutants. *Numbers above* the *bar* indicate the number of experiments. *, $p < 0.0001$. *C*, loss of specific binding to [^{3}H]RTX of the VR1 mutants, Δ^{114} and Δ^{761}, when transfected into HEK cells. *D*, the Δ^{114} and Δ^{761} mutants retained a heat-evoked current response comparable with that of the wild-type but lost the ability to produce acid-evoked currents. HEK cells transfected with the wild-type VR1, and the two VR1 mutants were treated with acid (pH 5.5; *filled bar*) or heat (46 °C; *open bar*). *Upper panel*, HEK cells transfected with the wild-type VR1 elicited a rapidly activating and inactivating current (*asterisk*) followed by a non-desensitizing current in response to acid (pH 5.5) application. In contrast, the Δ^{114} and Δ^{761} mutants showed rapidly activating and inactivating current only, which was presumably mediated by an acid-sensing ion channel (*ASIC*) endogenous to HEK cells, because it was observed in non-transfected cells. *Lower panel*, the two VR1 mutants elicited current responses to heat (46 °C), which were comparable with that of wild-type VR1.

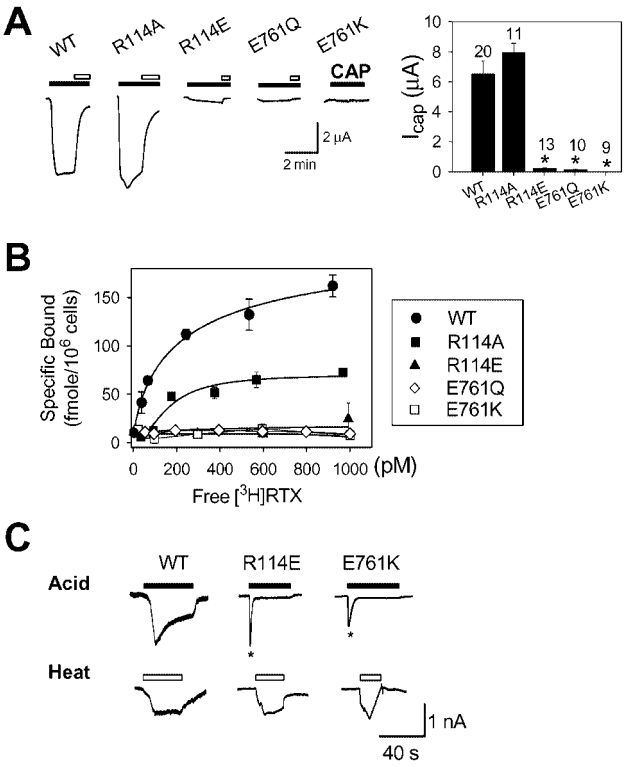

FIG. 6. **Changing charge of the two amino acids, Arg-114 and Glu-761, affects binding and current responses to vanilloids.** *A*, current responses to capsaicin of the VR1 mutants when expressed in *Xenopus* oocytes. Mutants with opposite charges at Arg-114 and Glu-761, *e.g.* R114E and E761K, lost I_{cap}. *Filled bars* represent the application of 1 μM capsaicin (*CAP*), and *open bars* represent the application of 10 μM capsazepine. *Right panel*, summary of I_{cap} of VR1 and its mutants. *Numbers above* the *bar* indicate the number of experiments. *, $p < 0.0001$. *B*, specific binding capacities of the VR1 mutants for [^{3}H]RTX. VR1 mutants, *e.g.* R114E and E761K, lost specific binding for [^{3}H]RTX ($n = 2\sim4$, triple determinations). *C*, the two mutants, R114E and E761K, retained heat-evoked (46 °C; *open bar*) but not acid-evoked (pH 5.5; *filled bar*) current response. *Asterisks* indicate rapidly activating and inactivating currents, presumably mediated by an ASIC channel endogenous to HEK cells.

recognition and that the positive charge at Arg-114 determines ligand binding to the channel but to a lesser extent than the charge at Glu-761.

DISCUSSION

VR1 is a molecular sensor present in small sensory neurons and is believed to be responsible for detecting various noxious stimuli. However, despite its physiological significance, the activation mechanisms of VR1 remain unknown. It has been reported that capsaicin and its analogs act on the intracellular side of the channel, suggesting the presence of ligand binding sites in the intracellular channel domains (28). Consistent with this idea, the present study identified two regions near Arg-114 and Glu-761 in the cytosolic tails of VR1 that determine ligand binding. These areas seemed to be minimal marginal sites required for channel activation, because truncation or point mutation of these regions causes a loss of capsaicin sensitivity and concomitant loss of [^{3}H]RTX binding capacity. Charges carried by the amino acids in the two regions appear to be minimal determinants of binding, because the introduction of neutral or oppositely charged residues at these positions causes a substantial to complete loss of ligand binding capacity. Based on the structure-activity relationships of synthetic capsaicin analogues (24, 25), it appears that the dipolar properties of phenolic hydroxide and of the amide group in capsaicin are important for its pharmacologic effects. In accordance with this proposition, it is highly conceivable that the charged residues at Glu-761, and at Arg-114 to a lesser extent, provide a hydrophilic interaction with capsaicin. Thus, in addition to the TM3 region in VR1, which is implicated in the hydrophobic interaction (27), the two areas in the cytosolic tails also constitute a part of the capsaicin binding pocket, as has been proposed by others (26).

Although the present study suggests that regions near the Arg-114 and Glu-761 residues in VR1 are key determinants of agonist binding, we cannot rule out the possibility that other regions of VR1 are also required to form a functional ligand

binding pocket. Indeed, Jordt and Julius (27) found that the TM3 region is also necessary for ligand binding, and this was confirmed by the present study (Fig. 1). Because the transmembrane domain region is embedded in a hydrophobic environment, the transmembrane region is believed to imply a hydrophobic interaction with VR1 (27). Furthermore, mutations of the sixth transmembrane domain were found to reduce [^{3}H]RTX binding affinity (38). Thus, it is conceivable that there are regions other than the two loci in the cytosolic tails or the third transmembrane domain that control VR1 ligand binding. Because we approached the sensitive areas by deleting segments of VR1 from N or C terminus, the presence of additional regions in the cytosolic tails located beyond Arg-114 and Glu-761 is also possible. More precise details of the ligand recognition sites, required for ligand binding, could be obtained by analyzing VR1 structurally.

Birds are not sensitive to vanilloids and can be fed hot chili peppers (26). Consistent with this, avian VR1 is insensitive to capsaicin (27). One plausible reason for the lack of capsaicin sensitivity of chick VR1 is that chick VR1 is equipped with a TM3 region that is not functional for capsaicin binding. When the TM3 region of chick VR1 is replaced with the cognate segment of rat VR1, the chick/rat VR1 chimera shows a sensitivity to capsaicin (27). This raises a question about the presence of the putative ligand recognition sites in the cytosolic tails in chick VR1. Indeed, chick VR1 has almost the same sequences in the putative ligand recognition sites as rat VR1 (see Fig. 2*A* and Fig. 3*A*). Therefore, chick VR1 appears to have a rudimentary backbone for vanilloid binding and elicits a rudimentary response to high doses of capsaicin (27). However, chick VR1 lacks only the functional TM3 region among the three minimal recognition sites. In contrast, VRL1, a capsaicin-insensitive homologue of VR1 with a relatively low sequence homology, failed to show current response to capsaicin even when a large segment of the transmembrane domain of VR1, including the TM3 region, was replaced (chimera *j* in Fig. 1). This is, in part, because VRL1 does not have functional ligand binding sites in the cytosolic tails. A VR1/VRL1 chimera is capsaicin-sensitive only when segments of VR1 containing the ligand binding regions in cytosolic tails and the TM3 regions are present. Thus, these results, together with those obtained using chick VR1, indicate that the putative ligand binding sites in the cytosolic tails are required for capsaicin sensitivity.

The activation of ligand-gated channels by ligands often requires interactions with several separate regions in the channel complexes. For example, the binding of glutamate to the *N*-methyl-D-aspartate receptor requires interaction between two extracellular domains, an extracellular region preceding the M1 transmembrane segment and an extracellular loop between the M3 and M4 segments (39). In contrast, the nicotinic acetylcholine receptor forms several distinct regions in the extracellular domains of its α- and non-α-subunits for ligand binding (40). In the present study, interaction between ligands and both regions in the N- and C-cytosolic tails appears to be obligatory for agonist binding, because the deletion of either of these two regions abrogates [^{3}H]RTX binding. The functional form of VR1 appears to be a homotetramer (17). Thus, vanilloids interact with the two putative regions within or between subunits of the VR1 channel complex when it is activated by capsaicin. To determine the nature of the possible receptor ligand interaction that occurs within or between VR1 subunits, we co-expressed two VR1 mutants, Δ^{1-114} and $\Delta^{761-838}$, each of which failed to respond to capsaicin when expressed alone. However, the co-expression of these two mutants failed to elicit I_{cap} or [^{3}H]RTX binding (data not shown). Because the two mutants contained each of the two obligatory regions, the failure of the co-expressed mutants to activate I_{cap} suggests that vanilloids interact with the two terminal regions of one VR1 subunit.

One striking feature of VR1 is its activation by heat and acid (3, 12). Moreover, VR1 activation has clinical implications, because ischemia or inflammation causes acidosis in tissues. In the present study, VR1 mutants, such as Δ^{114}, Δ^{761}, R114E, or E761K, lost their ability to produce capsaicin-sensitive currents but retained heat sensitivity (see Fig. 5*D* and Fig. 6*C*). These results indicate that the two regions in each cytosolic tail probably do not confer sensitivity to heat. Consistent with this, VRL1 is activated by heat but not by capsaicin. Therefore, activation mechanisms by heat and vanilloids appear distinct. Evidence now available suggests that the activation of VR1 by acid or capsaicin is mediated by distinct molecular mechanisms. The activation of VR1 by acid occurs only when the stimulus is applied to the extracellular surface of the channel, whereas activation by vanilloids occurs when from the intracellular side (12, 28). Mutations at charged residues on the extracellular surface or at a pore region of VR1 display reduced responses to acid as compared with capsaicin (29, 41). However, in the present study, deletion or disruption of the two sites in the cytosolic tails of VR1 dissipated capsaicin-sensitive currents and acid-gated responses (see Fig. 5*D* and Fig. 6*C*). Thus, unlike portions of the extracellular surface of VR1, these cytosolic regions appear to be linked functionally to the activation of VR1 by acid and by vanilloids.

In summary, in this study we identified minimal marginal regions in the cytosolic tails of VR1 that control its binding to ligands. Because the charges of the two amino acids in these regions appear critical to ligand binding, it is likely that these two obligatory sites share hydrophilic interaction with vanilloids. Thus, we believe that the present study provides the molecular elements of VR1 required for ligand binding. However, we cannot exclude the possible role of the intracellular domains in controlling folding or stabilization of the channel complex. The precise nature of the molecular interactions between VR1 and vanilloids should be determined by the structural analysis of VR1.

REFERENCES

1. Bevan, S., and Szolcsanyi, J. (1990) *Trends Pharmacol. Sci.* **11,** 330–333
2. Oh, U., Hwang, S. W., and Kim, D. (1996) *J. Neurosci.* **16,** 1659–1667
3. Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D., and Julius, D. (1997) *Nature* **389,** 816–824
4. Zygmunt, P. M., Petersson, J., Andersson, D. A., Chuang, H., Sorgard, M., Di Marzo, V., Julius, D., and Hogestatt, E. D. (1999) *Nature* **400,** 452–457
5. Hwang, S. W., Cho, H., Kwak, J., Lee, S.-Y., Kang, C. J., Jung, J., Cho, S., Min, K. H., Suh, Y. G., Kim, D., and Oh, U. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 6155–6160
6. Shin, J., Cho, H., Hwang, S. W., Jung, J., Shin, C. Y., Lee, S.-Y., Kim, S. H., Lee, M.-G, Choi, Y. H., Kim, J., Haber, N. A., Reichling, D. B., Khasar, S., Levine, J. D., and Oh, U. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 10150–10155
7. Premkumar, L. S., and Ahern, G. P. (1999) *Nature*, **408,** 985–990
8. Chuang, H.-H., Prescott, E. D., Kong, H., Shields, S., Jordt, S.-E., Basbaum, A. I., Chao, M. V., and Julius, D. (2001) *Nature* **411,** 957–962
9. Numazaki, M., Tominaga, T., Toyooka, H., and Tominaga, M. (2002) *J. Biol. Chem.* **277,** 13375–13378
10. LaMotte, R. H., and Campbell, J. N. (1978) *J. Neurophysiol.* **41,** 509–528
11. Meyer, R. A., and Campbell, J. N. (1981) *Science* **213,** 1527–1529
12. Tominaga, M., Caterina, M. J., Malmberg, A. B., Rosen, T. A., Gilbert, H., Skinner, K., Raumann, B. E., Basbaum, A. I., and Julius, D. (1998) *Neuron* **21,** 531–543
13. Caterina, M. J., and Julius, D. (2001) *Annu. Rev. Neurosci.* **24,** 487–517
14. Caterina, M. J., Leffler, A., Malmberg, A. B., Martin, W. J., Trafton, J., Petersen-Zeitz, K. R., Koltzenburg, M., Basbaum, A. I., and Julius, D. (2000) *Science* **288,** 306–313
15. Davis, J. B., Gray, J., Gunthorpe, M. J., Hatcher, J. P., Davey, P. T., Overend, P., Harries, M. H., Latcham, J., Clapham, C., Atkinson, K., Hughes, S. A., Rance, K., Grau, E., Harper, A. J., Pugh, P. L., Rogers, D. C., Bingham, S., Randall, A., and Sheardown, S. A. (2000) *Nature* **405,** 183–187
16. Harteneck, C., Plant, T. D., and Schultz, G. (2000) *Trends Neurosci.* **23,** 159–166
17. Kedei, N., Szabo, T., Lile, J. D., Treanor, J. J., Olah, Z., Iadarola, M. J., and Blumberg, P. M. (2001) *J. Biol. Chem.* **276,** 28613–28619
18. Smith, G. D., Gunthorpe, M. J., Kelsell, R. E., Hayes, P. D., Reilly, P., Facer,

P., Wright, J. E., Jerman, J. C., Walhin, J.-P., Ooi, L., Egerton, J., Charles, K. J., Smart, D., Randall, A. D., Anand, P., and Davis, J. B. (2002) *Nature* **418,** 186–190
19. Caterina, M. J., Rosen, T. A., Tominaga, M., Brake, A. J., and Julius, D. (1999) *Nature* **398,** 436–441
20. Liedtke, W., Choe, Y., Marti-Renom, M. A., Bell, A. M., Denis, C. S., Sali, A., Hudspeth, A. J., Friedman, J. M., and Heller, S. (2000) *Cell* **103,** 525–535
21. Suzuki, M., Sato, J., Kutsuwada, K., Ooki, G., and Imai, M. (1999) *J. Biol. Chem.* **274,** 6330–6335
22. Schumacher, M. A., Moff, I., Sudanagunta, S. P., and Levine, J. D. (2000) *J. Biol. Chem.* **275,** 2756–2762
23. Walpole, C. S., and Wrigglesworth, R. (1993) in *Capsaicin in the Study of Pain* (Wood, J. N., ed) pp. 63–82, Academic Press, San Diego, CA
24. Szolcsanyi, J., and Jancso-Garbor, A. (1975) *Arzneim. Forsch.* **25,** 1877–1881
25. Walpole, C. S., Wrigglesworth, R., Bevan, S., Campbell, E. A., Dray, A., James, I. F., Masdin, K. J., Perkins, M. N., and Winter, J. (1993) *J. Med. Chem.* **36,** 2373–2380
26. Szallasi, A., and Blumberg, P. M. (1999) *Pharmacol. Rev.* **51,** 159–212
27. Jordt, S.-E., and Julius, D. (2002) *Cell* **108,** 421–430
28. Jung, J., Hwang, S. W., Kwak, J., Lee, S.-Y., Kang, C. J., Kim, W. B., Kim, D., and Oh, U. (1999) *J. Neurosci.* **19,** 529–538
29. Welch, J. M., Simon, S. A., and Reinhart, P. H. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 13889–13894
30. Higuchi, R. (1989) in *PCR Technology*: *Principles and Applications for DNA Amplification* (Erlich, H. A., ed) pp. 61–70, Stokton, New York
31. Acs, G., Lee, J., Marquez, V. E., and Blumberg, P. M. (1996) *Brain Res. Mol. Brain. Res.* **35,** 173–182
32. Szallasi, A., and Blumberg, P. M. (1990) *Brain Res.* **524,** 106–111
33. Szallasi, A., Blumberg, P. M., Annicelli, L. L., Krause, J. E., and Cortright, D. N. (1999) *Mol. Pharmacol.* **56,** 581–587
34. Szallasi, A., Lewin, N. E., and Blumberg, P. M. (1992) *J. Pharmacol. Exp. Ther.* **262,** 883–888
35. Shin, J. S., Wang, M.-H., Hwang, S. W., Cho, H., Cho, S. Y., Kwon, M. J., Lee, S.-Y., and Oh, U. (2001) *Neurosci. Lett.* **299,** 135–139
36. Bevan, S., Hothi, S., Hughes, G., James, I. F., Rang, H. P., Shah, K., Walpole, C. S. J., and Yeats, J. C. (1992) *Br. J. Pharmacol.* **107,** 544–552
37. Gunthorpe, M. J., Smith, G. D., Davis, J. B., and Randall, A. D. (2001) *Pflugers Arch.* **442,** 668–674
38. Kuzhikandathil, E. V., Wang, H., Szabo, T., Morozova, N., Blumberg, P. M., and Oxford, G. S. (2001) *J. Neurosci.* **21,** 8697–8706
39. Laube, B., Hirai, H., Sturgess, M., Betz, H., and Kuhse, J. (1997) *Neuron* **18,** 493–503
40. Corringer, P.-J., Le Novere, N. L., and Changeux, J.-P. (2000) *Annu. Rev. Pharmacol. Toxicol.* **40,** 431–458
41. Jordt, S-E., Tominaga, M., and Julius, D. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 8134–8139