Identification of an Aspartic Residue in the P-loop of the Vanilloid Receptor That Modulates Pore Properties*

Vanilloid receptor subunit 1 (VR1) is a nonselective cation channel that integrates multiple pain-producing stimuli. VR1 channels are blocked with high efficacy by the well established noncompetitive antagonist ruthenium red and exhibit high permeability to divalent cations. The molecular determinants that define these functional properties remain elusive. We have addressed this question and evaluated by site-specific neutralization the contribution on pore properties of acidic residues located in the putative VR1 pore region. Mutant receptors expressed in Xenopus oocytes exhibited capsaicin-operated ionic currents akin to those of wild type channels. Incorporation of glutamate residues at Glu648 and Glu651 rendered minor effects on VR1 pore attributes, while Glu646 slightly modulated pore blockage. In contrast, replacement of Asp646 by asparagine decreased 10-fold ruthenium red blockade efficacy and reduced 4-fold the relative permeability of the divalent cation Mg2+ with respect to Na+ without changing the selectivity of monovalent cations. At variance with wild type channels and E636Q, E648Q, and E651Q mutant receptors, ruthenium red blockade of D646N mutants was weakly sensitive to extracellular pH acidification. Collectively, our results suggest that Asp646 is a molecular determinant of VR1 pore properties and imply that this residue may form a ring of negative charges that structures a high affinity binding site for cationic molecules at the extracellular entryway.

The molecular mechanism underlying chemical and thermal nociception is starting to be understood, thanks to the cloning of a capsaicin-operated neuronal receptor referred to as the vanilloid receptor subunit 1 (VR1)1 (1). VR1 is a nonselective cation channel with high Ca2+ permeability that integrates both types of pain-producing stimuli (1–5). These channels are activated by vanilloids such as capsaicin, the pungent ingredient of hot red peppers, and by temperatures higher than 40 °C (1, 2, 4). Recently, the lipid-based anandanamide was shown to be a potential endogenous VR1 agonist (6). Activation of the VR1 channel raises intracellular Ca2+ and excites a subset of dorsal root and trigeminal ganglion primary neurons (5). These nerves transmit nociceptive information to the central nervous system and release proinflammatory neuropeptides at peripheral terminals (5, 7). In addition to playing a role in nociception, the high Ca2+ permeability exhibited by VR1 strongly desensitizes capsaicin-operated responses (5, 7). This property partially accounts for the antinociceptive activity exhibited by vanilloids (5, 8, 9).

VR1 subunits are membrane proteins with a predicted relative molecular mass of 95 kDa that show similarity to the family of putative store-operated calcium channels (1, 3, 10). Although the molecular composition and stoichiometry of neuronal VR1 channels is undetermined, heterologous expression of VR1 subunits gives rise to homomeric receptors that recapitulate most of the reported physiological properties (1, 2, 4, 5, 7). Nonetheless, there is mounting evidence for molecular heterogeneity of vanilloid receptors, including the identification of a stretch-inactivating channel (11), a vanilloid receptor-like protein (VR1-1) (12), and an N-terminal splice variant of VR1 (VR.5 pv) (13).

Structurally, VR1 subunits display a hydrophilic intracellular N terminus domain containing three conserved ankyrin repeats and several kinase consensus sequences (Fig. 1). This protein domain might also contain the vanilloid binding site (13, 14). Hydrophobicity analysis of the protein reveals the presence of six putative transmembrane-spanning segments (S1 through S6) and a stretch linking the fifth and sixth segments that contains an amphipathic fragment denoted as the P-loop (Fig. 1). By analogy with shaker-like ion channels, this protein motif could critically contribute to the structure of the channel permeation pathway (15). Because this is a newly identified channel family, the molecular determinants that specify its pore attributes are yet unrecognized. Thus, their elucidation is a target of intense research. Amino acid sequence analysis of the VR1 putative pore region reveals the presence of four acidic residues, Glu636, Asp646, Glu648, and Glu651 (Fig. 1), that may play an important role defining the channel ion selectivity and blockade by noncompetitive antagonists such as ruthenium red (RR) (1, 2, 4, 5, 16). Recent evidence shows that mutation of Glu648 significantly reduced proton-activated currents without altering heat- or capsaicin-evoked responses or without eliminating the ability of protons to potentiate responses to these stimuli (17). Here, we report that site-specific neutralization of these negatively charged positions generated functional, capsaiacin-operated ion channels and show that the amino acid at position 646 modulates the RR inhibition efficacy. Further-

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¶¶ The abbreviations used are: VR1, vanilloid receptor subunit 1; RR, ruthenium red; GHK equation, Goldman-Hodgkin-Katz equation for the constant field approximation; pH∞, extracellular pH; NMG, N-methyl-D-glucamine.
more, our results show that neutralization of Asp^{646} significantly reduced the permeability of divalent cations with respect to Na\(^+\) without affecting that characteristic of monovalent cations. Taken together, these experiments support the tenet that Asp^{646} is a structural determinant of the VR1 pore-forming region.

**EXPERIMENTAL PROCEDURES**

Site-directed Mutagenesis, cRNA Preparation, and Microinjection into Xenopus Oocytes—VR1 is a cDNA clone encoding a functional capsaicin-operated channel from dorsal root ganglion (1) (kindly provided by Dr. David Julius). Site-directed mutagenesis was carried out by polymerase chain reaction as described (19, 20). Mutant receptors were confirmed by DNA sequencing. Capped cRNA was synthesized using the mMESSAGE mMACHINE in vitro transcription kit from Ambion (Austin, TX). 2–5 ng of cRNA was microinjected (V = 50 nl) into defolliculated oocytes (stages V and VI) as described (19). Oocytes were functionally assayed 3–5 days after cRNA injection.

Electrophysiological Characterization of the VR1 Mutants—Capsaicin-evoked whole cell currents were measured under voltage clamp (Turbo TEC 10CD; NPI Electronics, Tamm, FRG); oocytes were depolarized from 20 mV in 4 s (20 mV/s) unless otherwise indicated. Leak currents were measured in the absence of agonist in the external solutions that contain a short amphipathic fragment (curved arrow), and an intracellular C-terminal domain. On top is depicted the deduced amino acid sequence for the proposed pore-forming region (P-loop, underlined). Numbers on top denote the amino acid number in the deduced primary sequence.

**RESULTS**

Neutralization of Asp^{646} Reduces Ruthenium Red Sensitivity of VR1 Channels—To study the functional role played by negatively charged residues located within or nearby the proposed P-loop of VR1 channels (Fig. 1), we neutralized these acidic residues. Wild type and mutant receptors were expressed in Xenopus oocytes for functional characterization. Heterologous expression of VR1 transcripts in frog oocytes generated capsaicin-elicted ion currents that, at concentrations 10 \(\mu\)M, activated slowly to reach a relatively stable plateau level and subsided rapidly to base line upon agonist washout (Fig. 2A). Higher vanilloid concentrations accelerated receptor activation and concomitantly delayed agonist removal (Fig. 2B). These capsaicin-operated responses exhibited an EC_{50} (concentration of agonist to activate half-maximal response) of \(-0.5 \mu\)M, in agreement with other reports (1, 2). Neutralization of acidic residues in the pore region did not significantly modify capsaicin efficacy (EC_{50} \(\sim\) 0.2–0.5 \(\mu\)M for E636Q, D646N, E648Q, and E651Q mutants); nor did it change dramatically the maximal current (Table I). Activation and desensitization were altered by the mutations performed (data not shown).

To investigate whether the mutated amino acids modulate pore properties, we used as a sensitive probe the positively charged ruthenium red, a VR1 channel blocker. These studies were performed at saturating vanilloid concentrations to ensure complete and fast channel opening. We choose 20 \(\mu\)M...
Molecular Determinants of VR1 Pore Function

Fig. 2. Neutralization of acidic residues modulates receptor sensitivity to RR inhibition. Ionic currents evoked by 10 μM (A) and 20 μM (B) capsaicin from *Xenopus* oocytes expressing RNA transcripts of VR1. C–F, ruthenium red blockade on wild type and mutant receptor. Oocytes were bathed in Mg
-Ringer’s solution and activated with 20 μM capsaicin in the absence or presence of increasing blocker concentrations. Membrane currents were recorded in the whole-cell voltage clamp configuration, at V0 = −80 mV. Capsaicin and blocker were applied for the duration indicated by the horizontal bars. Maximal currents elicited by capsaicin were as follows: 390 ± 678 nA (number of oocytes (N) = 128) for wild type; 319 ± 222 (N = 61) for E636Q; 574 ± 644 (N = 74) for D646N; 410 ± 503 (N = 37) for E648Q; 118 ± 123 (N = 20) for E651Q. Values are given as mean ± S.D. **p < 0.1 as compared with wild type.

capsaicin because it evoked a rapid activation to a plateau level that readily declined to the original base line upon agonist washout (Fig. 2B). As illustrated in Fig. 2C, oocytes expressing wild type channels exhibited capsaicin-operated ionic currents that were rapidly blocked in a concentration-dependent manner by micromolar RR concentrations applied extracellularly. RR inhibition of VR1 channels was washable and weakly voltage-dependent (data not shown). Neutralization of Glu636 and Glu646 to glutamine gave rise to functional channels that responded to capsaicin and displayed RR sensitivity similar to that characteristic of wild type receptors (Fig. 2D and F). Similar results were obtained for E651Q (Table I). In contrast, charge neutralization of Asp646 (D646N) generated channels that were significantly less sensitive to RR than wild type receptors (Fig. 2E). Whereas VR1 channels were blocked by 80% with 1 μM RR, capsaicin-elicted responses from D646N mutants were only reduced by 25%. Complete blockade of D646N channels required RR concentrations as high as 50 μM.

RR sensitivity of VR1 species was quantified from dose-response relationships (Fig. 3, top, and Table I). RR blocked VR1 wild type channels with an IC50 of 0.14 ± 0.09 μM and a steepness, n, of 0.9 ± 0.3. Neutralization of Glu646 and Glu651 did not significantly alter block RR blockade efficacy (IC50 = 0.25 ± 0.06 μM for E648Q, and IC50 = 0.16 ± 0.03 μM for E651Q). Replacement of E646 by glutamine, however, increased the RR blockade efficacy by ~3-fold (IC50 = 0.04 ± 0.01 μM). In contrast, mutation of Asp646 to asparagine reduced RR sensitivity by ~10-fold (IC50 = 1.7 ± 0.2 μM). These data suggest that Asp646 is a molecular determinant of RR sensitivity and that the residue at position 636 modulates pore blockade. Replacement of the acidic residues by lysine at these positions (E636K and D646K) or simultaneous neutralization of both residues, E636Q/D646N, did not produce functional capsaicin-operated channels, precluding a detailed study of the functional interplay of these two protein positions.

Acidification of the Extracellular Medium Modulates RR Blockade Efficacy—The stability of a complex between RR and pore acidic residues will be determined by their degree of protonation and the spatial arrangement of carboxylic groups. Accordingly, protonation of acidic groups involved in RR binding should weaken its blockade efficacy. We tested this prediction by obtaining the RR inhibition efficacy for all VR1 species at acidic extracellular pH (pH). As illustrated in Fig. 3 (bottom), VR1 wild type and E636Q, E648Q, and E651Q mutant receptors exhibited ~10-fold lower sensitivity to RR blockade at pH 6.4 (Table I) as compared with the neutral pH 7.4. In marked contrast, extracellular acidification reduced by only 2-fold RR blockade efficacy of D646N mutants (Table I and Fig. 3, bottom). These observations suggest that carboxylate groups involved in RR binding become protonated at pH 6.4, resulting in a reduced RR blocking sensitivity. That carboxylate groups exhibit rather neutral pKa values is not surprising, since pKa of acidic groups buried inside proteins can vary several units depending on the dielectric environment (25), as evidenced for Ca2+-channels and cyclic GMP-gated channels (26, 27). These studies

| Species               | pH 7.4 | pH 6.4 |
|-----------------------|--------|--------|
|                       | IC50  | IC50  |
|                       | μM    | μM    |
| VR1                   | 0.14  | 0.13  |
| E636Q                 | 0.04  | 0.03  |
| D646N                 | 1.7   | 3.0   |
| E648Q                 | 0.25  | 1.5   |
| E651Q                 | 0.16  | 1.3   |
| M644Y                 | 0.20  | ND    |
| E636K/K639E           | 0.17  | 0.9   |

Table I

Ruthenium red blockade efficacy of VR1 channels

Experimental values were fitted to the logistic equation: $I_{\text{blocker}}/I_{\text{agonist}} = 1/(1 + (\text{blocker}/IC_{50})^f)$, where $IC_{50}$ denotes the [blocker] to inhibit half of the maximal agonist response, and $n$ is the steepness of the relationship. E636K and D646K did not exhibit capsaicin-evokedionic currents. Data are given as mean ± S.E., with $N > 3$. ND, not determined.
Molecular Determinants of VR1 Pore Function

D646N Mutant Channels Display Lower Permeability to Mg$^{2+}$—Since Asp$^{646}$ appears to be an important structural determinant of VR1 channel blockade, it is conceivable that this residue also contributes to define the ionic permeability, especially to divalent cations. To test this hypothesis, we investigated the relative ionic permeability of wild type and D646N mutant channels. We focused on Mg$^{2+}$ for two reasons: (a) Mg$^{2+}$ is not an activator of the endogenous calcium-activated chloride conductance (24); and (b) at variance with Ca$^{2+}$, Mg$^{2+}$ does not block nor desensitize VR1 channels at millimolar concentrations. Indeed, attempts to measure the relative Ca$^{2+}$ permeability failed due to the large blockade and desensitization of VR1 channels provoked by this cation, consistent with previous observations (1, 2). Capsaicin-evoked ionic currents from VR1 wild type channels in the presence of [Ca$^{2+}$]o ≈ 1 mM were ≈10 nA, preventing the accurate measurement of reversal potentials.

For VR1 wild type channels, I-V relationships obtained at low [sodium]o (20 mM) and 1 mM MgCl$_2$ are fairly linear, with a slight inward rectification at negative membrane potentials, and exhibit a reversal potential ($V_r$) of $-36 \pm 3$ mV (Fig. 4A). A 10-fold increase in [Mg$^{2+}$]o shifted the reversal potential up to 25 mV toward more depolarizing potentials (Fig. 4A), indicating that VR1 channels are permeable to Mg$^{2+}$. Mutation of Asp$^{646}$ to asparagine slightly affected the I-V characteristics but significantly altered the permeability to Mg$^{2+}$ (Fig. 4B). As shown, a 10-fold increase in [Mg$^{2+}$]o, moved $V_r$, $-10$ mV toward positive potentials. This displacement of $V_r$ was 15 mV smaller than that observed for VR1 channels, suggesting that the D646N mutant receptor exhibits lower permeability to Mg$^{2+}$. At variance with the D646N mutant, neutralization of the other negatively charged residues did not significantly affect the apparent Mg$^{2+}$ permeability, as evidenced by the similar shift in $V_r$ consequent to changing the extracellular ionic conditions (Table II). Analysis of I-V relationships obtained varying the [Na$^+$]o suggested that the permeability to monovalent cations was unaffected by the mutations (Table II).

To further underscore that Asp$^{646}$ is a molecular determinant of VR1 ionic selectivity, we determined the relative permeabilities to K$^+$ and Mg$^{2+}$ with respect to Na$^+$, using the

![Figure 4](image-url)
Molecular Determinants of VR1 Pore Function

TABLE II
Reversal potentials of VR1 mutants

| Species     | V_r (mV) | ΔV_r (mV) |
|-------------|----------|-----------|
| VR1         | 6 ± 4    | 6 ± 2     |
| E636Q       | 4 ± 3    | 4 ± 2     |
| D646N       | −2 ± 4   | −3 ± 3    |
| E648Q       | 7 ± 5    | 6 ± 4     |
| E651Q       | 3 ± 4    | 3 ± 5     |
| M644Y       | 1 ± 2    | 2 ± 2     |
| E636K/K639E | 3 ± 1    | 3 ± 3     |

a p < 0.05.

GHK equation modified to include the contribution of divalent cations (22, 23). Reversal potentials were plotted as a function of the external Mg²⁺ activities, and the experimental data were fitted to the GHK equation (Fig. 4C). For wild type channels, the parameters that best fit the data were $P_{K^+/P_{Na^+}} = 0.9 ± 0.1$ and $P_{Mg²⁺/P_{Na^+}} = 4.0 ± 0.3$, which are in good agreement with those reported by others (1). This result implies that VR1 channels display a similar selectivity to $K^+$ and $Na^+$ and a preferential permeability for Mg²⁺ over $Na^+$. Neutralization of Asp⁶⁴⁶ with asparagine decreased 4-fold the $P_{Mg²⁺/P_{Na^+}} (1.0 ± 0.2)$ without significantly affecting the permeability to monovalent cations ($P_{K^+/P_{Na^+}} = 0.8 ± 0.2$). Thus, these findings using Mg²⁺ as a divalent cation hint that Asp⁶⁴⁶ contributes to the permeability to divalent cations.

**Mutation of Met⁶⁴⁴ to Tyrosine Appears Not to Be Essential to Define Pore Properties**—The Asp⁶⁴⁶ residue is located in the sequence motif TXGMGD, which is virtually identical to the K⁺ channel signature sequence TXGYGD (15). To further investigate the role of this amino acid sequence in pore properties, we mutated Met⁶⁴⁴ to tyrosine. The M644Y mutant channel was functional and exhibited capsaicin-operated ionic currents in *Xenopus* oocytes (Fig. 5A). The capsaicin EC₅₀ was not changed by the mutation (−0.3 μM). Likewise, the incorporation of a tyrosine in Met⁶⁴⁴ did not alter the RR sensitivity (Table I). However, this mutant channel exhibited a remarkably slow kinetics of the capsaicin-operated responses (Fig. 5C), suggesting that the amino acid at this position may modulate channel gating.

We also studied the relative ionic permeability of $K^+$ and $Mg²⁺$ to $Na^+$. As illustrated in Table II, a 6-fold increase in the [Na]₀ shifted the reversal potential by 29 mV, while a 2.5-fold increment in the [Mg²⁺]₁ changed $V_r$ up to 7 mV toward depolarizing potentials. These data suggest that replacement of Met⁶⁴⁴ by tyrosine slightly modified the permeation properties.

To further support this notion, we determined the relative ionic permeabilities to $K^+$ and $Mg²⁺$ with respect to $Na^+$ using the GHK approximation. The estimated ionic permeabilities were $P_{K^+/P_{Na^+}} = 1.1 ± 0.1$ and $P_{Mg²⁺/P_{Na^+}} = 2.9 ± 0.4$. These results indicate that, at least modest 20% increase and 30% decrease in $K^+$ and $Mg²⁺$ permeability, respectively. Collectively, these data indicate that the residue at position 644 plays a marginal role in modulating the ionic permeability, similar to the function assigned to this amino acid in *shaker*-like K⁺ channels (30).

**Mutation of Lys⁶³⁹ to Glutamic Acid**—The proposed similar modular organization of VR1 and *shaker*-like channels implies a comparable pore structure composed of a selectivity filter and a pore helix (15). The pore helix would encompass residues from Asn⁶²⁵ to Phe⁶⁴⁰ and contain residue Glu⁶⁴⁶, which could form an intrahelical salt bridge with Lys⁶³⁹ (Fig. 1). Incorporation of a positively charged residue at position Glu⁶⁴⁶ (E636K) renders nonfunctional VR1 channels (Fig. 5B), presumably by disrupting this interaction. Should be this the case, mutation of Lys⁶³⁹ to Glu in the mutant E636K can rescue channel function. We tested this hypothesis, and, in contrast to E636K (Fig. 5B), heterologous expression of the double mutant in Xenopus oocytes gave rise to capsaicin-operated ionic currents that closely resemble those of wild type channel (Fig. 5C). Indeed, the capsaicin EC₅₀ for this mutant is analogous to wild type channels (data not shown). Furthermore, the E636K/K639E double mutant exhibits its RR sensitivity (Fig. 5D, Table I) and ionic permeability (Table I) similar to VR1 wild type. Thus, the E636K/K639E double mutant recapitulates the functional pore properties of wild type channels. This result is consistent with existence of a
Molecular Determinants of VR1 Pore Function

Homomeric VR1 channels expressed in Xenopus oocytes gave rise to capsaicin-activated ionic currents sensitive to RR inhibition and to receptors permeable to divalent cations. Site-specific neutralization of acidic residues in the putative pore-forming region influenced the pore attributes of homomeric VR1 channels. The most salient contribution of these studies is the identification of the amino acid at position 646 (Fig. 1) as a molecular determinant of pore properties such as blockade by ruthenium red and Mg\(^{2+}\)-permeability. Replacement of Asp\(^{646}\) by asparagine created channels exhibiting lower sensitivity to RR blockade and reduced Mg\(^{2+}\)-permeability with respect to Na\(^+\). Charge neutralization of Glu\(^{648}\) and Glu\(^{651}\) residues did not alter these VR1 pore properties, while mutation of Glu\(^{650}\) weakly modulated pore blockade. Modulation of both channel blockade and Mg\(^{2+}\)-permeability by Asp\(^{646}\) suggests a direct interaction of RR and the divergent cation with this residue and implies that the spatial arrangement of carboxylic groups structures a high affinity binding site for cationic molecules in the pore, similar to that described for Ca\(^{2+}\)-permeable channels (26, 27, 31–33). It should be noted, however, that neutralization of Asp\(^{646}\) neither prevented completely RR blockade nor drastically changed the ionic selectivity, suggesting the contribution of other amino acids determining these pore properties. Consistent with this view, incorporation of a glutamate at position 636 increased 3-fold RR inhibition efficacy, suggesting a role of this amino acid in modulating pore blockade, perhaps by tuning the geometry of the blocker binding site. The double mutant E636Q/D646N, which could have further assisted in understanding the interplay of these two protein positions defining pore function did not generate capsaicin-activated channels, precluding any functional characterization.

Although caution must be exercised when inferring protein structure from functional assays using site-directed mutagenesis, our data identify the P-loop on VR1 as a basic pore module that governs key properties of ion permeation and pore blockade. The proposed molecular model for VR1 channel resembles that established for shaker type channels; namely they encompass six transmembrane-spanning segments, representing the S5–P–S6 region of the pore module (Fig. 1). The high resolution structure of a bacterial K\(^+\) channel (KcsA) from Streptomyces lividans, formed by only two transmembrane segments and a connecting amphipathic loop, has provided fundamental insights into the mechanisms underlying pore function (15). Our functional observations endorse the tenet that VR1 channels exhibit a similar modular organization as K\(^+\) channels, consistent with a model in which a ring of presumably four P-loops forms the inner core of the channel. This claim is further underscored by comparison of the amino acid sequences of KcsA and VR1 channels. In particular, there are seven residues in the C-end half of the P-loop that are virtually identical in both channel types, including the signature sequence motif TXGYGD that in VR1 channels is TXGMGD, thus suggesting a similar pore organization. A central question remains: is the spatial location of acidic residues in the VR1 channel consistent with their role in pore function? In analogy to the KcsA channel, the pore in the VR1 channel would be composed of a turret, pore helix, and selectivity filter (15). This architecture would place Glu\(^{630}\) in the pore helix, Asp\(^{646}\) at the pore entrance, and Glu\(^{648}\) and Glu\(^{651}\) would be nearby the extracellular entryway. The finding that the nonfunctional phenotype of the E636K mutant can be efficiently rescued by the additional mutation of Lys\(^{639}\) to glutamic acid (E636K/K639E) is compatible with the tenet that these charged residues form an intrahelical salt bridge and, in turn, suggests that the segment comprising from Asn\(^{625}\) to Phe\(^{640}\) may have α-helical secondary structure (Fig. 1). Accordingly, the location of Glu\(^{636}\) would be consistent with a role providing stability to the channel selectivity filter and/or contributing to hold the putative tetramer together (15). In contrast, Asp\(^{646}\) could structure a high affinity cation binding site right at the pore vestibule by strategically positioning a ring of carboxylate groups. In support of this view, substituted cysteine accessibility studies in the carboxyl half of the P region of shaker-like K\(^+\) channels, together with the crystallographic structure of KcsA, reveal that the acidic residue in the sequence motif TXGYGD is exposed at the outer mouth of the channel (15, 34). A symmetric distribution of aspartic residues at the extracellular entryway would be essential to coordinate cationic molecules (25), thus modulating ion permeation and blockade in this channel family. Positioning two additional acidic residues, Glu\(^{646}\) and Glu\(^{651}\), close to the permeation pathway will ensure a strong negative electrostatic potential required to raise the local concentration of positively charged molecules such as ruthenium red and cations.

To further substantiate the proposed pore organization, we mutated Met\(^{644}\) in the VR1 sequence motif TXGMG to tyrosine (Fig. 1). The M644Y mutant channel exhibited capsaicin-activated ionic currents that were sensitive to RR block. The relative ionic permeability to K\(^+\) and Mg\(^{2+}\) with respect to Na\(^+\) was slightly lower than that characteristic of wild type channels, implying a marginal role of this residue in modulating ionic selectivity. This finding is in good agreement with studies in shaker-like K\(^+\) channels, showing that nonconservative mutations of the aromatic residue at this position (TXGYGD) leave the K\(^+\) selectivity intact (30). Indeed, the tyrosine side chains point away from the pore and make interactions with residues from the helix pore (15). It is noteworthy that, in both VR1 and shaker-like K\(^+\) channels, mutations at this position markedly affect channel gating (30). Additional work is necessary to understand the role of this amino acid in channel function.

Collectively, our findings imply that the underlying pore-forming region of VR1 shares structural features of the well known shaker-like K\(^+\) channels and indicate that Asp\(^{646}\) modulates VR1 pore properties. Nonetheless, the amino acid at this position is not sufficient to account for all pore properties of VR1, therefore suggesting the existence of additional structural determinants. Accordingly, it is plausible that amino acid residues at the extracellular end of S5 and S6 located near the membrane-water interface as well as amino acids in the linkers that connect these segments with the P-loop modulate pore attributes, as reported for cyclic GMP-gated ion channels (26). Likewise, a contribution of nonacidic pore residues cannot be ruled out (35). Further studies are needed to identify additional molecular determinants and to decipher the molecular mechanisms implicated in VR1 pore function. We have initiated a substituted cysteine accessibility reporter strategy to unveil the pore structure at the inner and outer surfaces. The proposed model should provide a testable hypothesis that may contribute to outline a molecular blueprint for the pore-forming region of this newly identified channel family.

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REFERENCES

1. Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D., and Julius, D. (1997) Nature 389, 816–824
2. Tominaga, M., Caterina, M. J., Malmberg, A. B., Rosen, T. A., Gilvert, H., Skinner, K., Raumann, B. E., Bisbaum, A. I., and Julius, D. (1998) Neuron 21, 531–543
3. Caterina, M. J., and Julius, D. (1998) Curr. Opin. Neurobiol. 9, 525–530
4. Nagy, I., and Rang, H. P. (1999) J. Neurosci. 19, 10647–10665
5. Szallasi, A., and Blumberg, P. M. (1999) Pharmacol. Rev. 51, 159–211
6. Zygmunt, P., Peterson, J., Andersson, D., Chuang, H., Sørgård, M., Di Marzo, V., Julius, D., and Högestätt, E. (1999) Nature 400, 452–457
7. Wood, J. N. (ed) (1993) Capsaicin in the Study of Pain, Academic Press, Inc., San Diego
8. Sterner, O., and Szallasi, A. (1999) Trends Pharmacol. Sci. 20, 459–465
9. Williams, M., Kowaluk, E. A., and Arneric, S. P. (1999) J. Med. Chem. 42, 1481–1500
10. Birnbaumer, L., Zhu, X., Jiang, M., Boulay, G., Peyton, M., Vannier, B., Brown, D., Platano, D., Sadeghi, H., Stefani, E., and Birnbaumer, M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 15196–15202
11. Suzuki, M., Sato, J., Kutsuwada, K., Ooki, G., and Imai, M. (1999) J. Biol. Chem. 274, 6329–6335
12. Caterina, M. J., Rosen, T. A., Tominaga, M., Brake, A. J., and Julius, D. (1999) Nature 398, 436–441
13. Schumacher, M. A., Moff, I., Sudanagunta, S. P., Levine, J. D. (2000) J. Biol. Chem. 275, 2766–2762
14. 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, 529–536
15. Doyle, D. A., Cabral, J. M., Pfuetzner, R. A., Kuo, A., Gulbis, J. M. Cohen, S. L., Chait, B. T., and MacKinnon, R. (1998) Science 280, 68–77
16. Dray, A., Forbes, C. A., and Burgess, G. M. (1999) Nature 398, 525–529
17. Jort, S.-V., Tominaga, M., and Julius, D. (2000) Proc. Natl. Acad. Sci. 97, 8134–8139
18. Ferrer-Montiel, A. V., Garcia, C. Morenilla, C. Merino, J. M., and Planells-Cases, R. (2000) Eur. J. Neurosci. 12, 124 (Abstr. 059.11)
19. Ferrer-Montiel, A. V., and Montal, M. (1994) Methods Companion Methods Enzymol. 6, 60–69
20. Ferrer-Montiel, A. V., and Montal, M. (1999) Methods Mol. Biol. 128, 167–178
21. Ferrer-Montiel, A. V., Sun, W., and Montal, M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8021–8025
22. Ferrer-Montiel, A. V., Sun, W., and Montal, M. (1996) Biophys. J. 71, 749–758
23. Lewis, C. A. (1979) J. Physiol. 280, 68–77
24. Miledi, R., and Parkin, P. (1985) J. Physiol. 357, 173–183
25. Feesht, A. (1985) Enzyme Structure and Mechanism, Freeman & Co., New York
26. Seifert, R., Eismann, E., Ludwing, J., Baumann, A., and Kaupp, U. B. (1999) EMBO J. 18, 119–130
27. Klockner, Mikala, G., Schwartz, A., and Varady, G. (1996) J. Biol. Chem. 271, 22293–22296
28. Bas, H., and Tora, H. (1992) The Chemistry of Acid Derivatives, Suppl. B, Vol. 2, pp. 1–50, John Wiley & Sons, Inc., New York
29. Zalaweski, R. I. (1992) The Chemistry of Acid Derivatives, Suppl. B, Vol. 2, pp. 305–369, John Wiley & Sons, Inc., New York
30. Heginbotham, L., Lu, Z., Abramson, T., and MacKinnon, R. (1994) Biophys. J. 66, 1061–1067
31. Ellinor, P. T., Yang, J., Sather, W. A., Zhang, J.-F., and Tsien, W. A. (1995) Neuron 14, 121–123
32. Premkumar, L. S., and Auerbach, A. (1998) Neuron 15, 869–880
33. Beck, C. A, Woolmuth, L. P., Seeburg, P. H., Sakmann, B., and Kuner, T. (1999) Neuron 22, 559–570
34. Pascual, J. M., Shiieh, C.-C., Kirsch, G. E., and Brown, A. M. (1995) Neuron 2, 1055–1063
35. Williamson, A. V., and Sather, W. A. (1999) Biophys. J. 77, 2575–2589