We have studied the molecular determinants of ion permeation through the TRPV4 channel (VRL-2, TRP12, VR-OAC, and OTRPC4). TRPV4 is characterized by both inward and outward rectification, voltage-dependent block by Ruthenium Red, a moderate selectivity for divalent versus monovalent cations, and an Eisenman IV permeability sequence. We identify two aspartate residues, Asp672 and Asp682, as important determinants of the Ca\(^{2+}\) sensitivity of the TRPV4 pore. Neutralization of either aspartate to alanine caused a moderate reduction of the relative permeability for divalent cations and of the degree of outward rectification. Neutralizing both aspartates simultaneously caused a much stronger reduction of Ca\(^{2+}\) permeability and channel rectification and additionally altered the permeability order for monovalent cations toward Eisenman sequence II or I. Moreover, neutralizing Asp682 but not Asp672 strongly reduces the affinity of the channel for Ruthenium Red. Mutations to Met680, which is located at the center of a putative selectivity filter, strongly reduced whole cell current amplitude and impaired Ca\(^{2+}\) permeation. In contrast, neutralizing the only positively charged residue in the putative pore region, Lys675, had no obvious effects on the properties of the TRPV4 channel pore. Our findings delineate the pore region of TRPV4 and give a first insight into the possible architecture of its permeation pathway.

MATERIALS AND METHODS

**Cell Culture and Molecular Biology**—We used the recombinant bicistronic expression plasmid pdTRP12, which carries the entire protein-coding region for murine TRPV4 (mouse mTRP12; accession number CAC20703) and for green fluorescent protein coupled by an IRES sequence. Human embryonic kidney cells, HEK293, were grown in Dulbecco’s modified Eagle’s medium containing 10% (v/v) human serum, 1 mM MgCl\(_2\), 5 mM CaCl\(_2\), 10 mM glucose, 10 mM HEPES, buffered at pH 7.4 with NaOH. The osmolality of this solution, as measured with a vapor pressure osmometer (Wescor 5500, Schlag, Gladbach, Germany), was 320 ± 5 mosmol. When indicated in the figure legends, the Ca\(^{2+}\) concentration of this solution was varied between 0 and 30 mM. To study the relative permeability of monovalent and divalent cations, we used extracellular solutions containing 1 mM MgCl\(_2\), 10 mM glucose,

**Solutions**—The standard extracellular solution contained 150 mM NaCl, 1 mM MgCl\(_2\), 5 mM CaCl\(_2\), 10 mM glucose, 10 mM HEPES, buffered at pH 7.4 with NaOH. The osmolality of this solution, as measured with a vapor pressure osmometer (Wescor 5500, Schlag, Gladbach, Germany), was 320 ± 5 mosmol. When indicated in the figure legends, the Ca\(^{2+}\) concentration of this solution was varied between 0 and 30 mM. To study the relative permeability of monovalent and divalent cations, we used extracellular solutions containing 1 mM MgCl\(_2\), 10 mM glucose,
Pore Mutations in TRPV4

Fig. 1  Alignment of the putative TRPV4 pore region with that of other TRPV channels and of KcsA. Transmembrane topology of TRPV channels (top) and alignment of the putative pore region of TRPV channels and KcsA (bottom). The grey area marks the region with the highest homology between for TRPV1, TRPV2, and TRPV4. Charged residues within this region are marked with bold type. The positions of the specific TRPV4 activator 4α-phorbol 12,13-didecanoate (4α-PDD; Sigma) are indicated on top. GenBank® accession numbers are CAC 20703 (TRPV4), CAB 89866 (TRPV1), NP 057197 (TRPV2), NP 062815 (TRPV5), AAG 41951 (TRPV6), and PIR S60172 (KcsA). A, ankyrin-binding repeats.

TRPV4 SETSFAGLLDFKLITIGMCDELEML
TRPV1 YNSLYSTCLDFKFITGMCDELEFT
TRPV2 YRGLIESLDKFKFTIGMCELAFQ
TRPV5 YPTALFLSTFELF-ITIIDGPNY
TRPV6 YPMALFSTFELF-ITIIDGPNYD
KcsA YPRALWWSVETATTVGYCGDLYPV

RESULTS

Pore Mutants of TRPV4 Yield Functional Channels—Fig. 1 shows an alignment of the putative pore region of murine TRPV4 (residues 663–686) with the corresponding regions of the other members of the TRPV subfamily and of the bacterial potassium channel KcsA. In this region, TRPV4 has the highest homology with TRPV1 and TRPV2, with which it shares a central stretch of 11 highly conserved amino acids. There is also significant homology with TRPV5 and TRPV6 and, to a lesser extent, with the pore region of KcsA. When comparing TRPV1/2/4 with the highly Ca2+-selective TRPV5/TRPV6 pores two differences are immediately obvious. Firstly, the TRPV1/2/4 pores contain a positively charged lysine (Lys675 in TRPV4), which is not present in TRPV5/6. Secondly, TRPV1/2/4 contain a methionine (Met680 in TRPV4) at the position corresponding to Asp542 in TRPV5 (Asp541 in TRPV6), which is crucial for the high Ca2+ selectivity of the latter channel. Moreover, TRPV4 contains two negatively charged residues within the conserved stretch of 11 amino acids: Asp542, which is conserved among all TRPVs, and Asp540, which is not present in TRPV5/6 and corresponds to Asp542 in TRPV1, a residue involved in Ruthenium Red block and Mg2+ permeability. To assess the contribution of these TRPV4 pore residues to the pore properties of the channel, we constructed seven cDNAs coding for TRPV4 channels in which point mutations were introduced into the pore. Charged residues were neutralized (mutants D672A, D682A, and K675A and double mutant D572A/D682A), the pore methionine residue was substituted by an alanine (mutant M680A), and, to mimic the TRPV5/6 pore, a negative charge was introduced, either alone or in combination with a deletion of the positively charged Lys675 (mutants M680D and M680D/K675D). As shown in Fig. 2, all seven mutants expressed as functional ion channels, as indicated by the activation of a cation current upon stimulation of transient HEK293 cells with the specific TRPV4 activator 4α-PDD. When compared with wild type TRPV4, the current densities were significantly larger for the D572A, D582A, and D572A/D582A mutants, whereas mutations to Met680 led to a reduction in current density. These differences in current density could arise from alterations in plasma membrane expression, single-channel conductance, or sensitivity toward activation by 4α-PDD. This latter possibility would not be unprecedented, because gating of TRPV1 by extracellular protons critically depends on a negatively charged residue (Glu462) within the TRPV1 pore region (17), but more work is needed to elucidate.

Electrophysiological Recordings—Whole cell membrane currents were monitored with an EPC-9 (HEKA Elektronik, Lambrecht, Germany; 8-Pole Bessel filter, 2.9 kHz) using ruptured patches. An Ag-AgCl wire was used as a reference electrode. Capacitance and access resistance were monitored continuously. Between 50 and 70% of the series resistance was electronically compensated to minimize voltage errors. Unless mentioned otherwise, we have applied a ramp protocol consisting of a 20-ms voltage step to −100 mV followed by a 380-ms linear ramp to +100 mV. This protocol was repeated every 5 s. The currents were sampled at 1.25 kHz. The time course of the whole cell current and current densities were obtained by averaging the current in a narrow window around ~80 mV during the voltage ramp protocol. All of the experiments were performed at room temperature (20–23 °C).

Calculation of the Relative Permeability of Mono- and Divalent Cations—The relative permeability of monovalent cations was calculated from the shift in reversal potential after complete substitution of extracellular Na+ by the specific cation, according to the following equation.

\[ \frac{P_X}{P_{Na}} = \exp(\Delta V_{rev} \times RT) \]  

(Eq. 1)

where \( \Delta V_{rev} \) is the measured shift in reversal potential. Permeability of the divalent cations Ca2+ and Mg2+ relative to Na+ was calculated from the absolute reversal potential measured with 30 mM of the respective cation in the extracellular solution, according to the following equation.

\[ \frac{P_X}{P_{Na}} = \left(1 + \exp(\Delta V_{exp} \times RT)\right)^{-1} \]  

(Eq. 2)

where \( P_X \) represents the permeability of the divalent cation, \( [X] \), represents its extracellular concentration, \( \alpha \) is \( P_{X}/P_{Na} \) obtained from Equation 1, \( [Na]^+ \), \( [Na]^- \), \( [Cs]^+ \), and \( [Cs]^- \), are the extra- and intra-cellular concentrations for Na+ and Cs+, respectively, and \( V_{ori} \), represents the reversal potential (9–11). Before calculation of the relative permeabilities, all reversal potentials were corrected for liquid junction potentials (15).

\[ E_{corrected} = E_{measured} - V_{LJ} \]  

(Eq. 3)

where \( V_{LJ} \), the liquid junction potential, was calculated according to Barry (16). Note that the current-voltage relations in Figs. 2–5 are not corrected for liquid junction potential.

Statistical Analysis—The data are expressed as the means ± S.E. Overall statistical significance was determined by analysis of variance. In case of significance (p < 0.01), individual groups were compared using Student’s t test.

RESULTS

Pore Mutants of TRPV4 Yield Functional Channels—Fig. 1 shows an alignment of the putative pore region of murine TRPV4 (residues 663–686) with the corresponding regions of the other members of the TRPV subfamily and of the bacterial potassium channel KcsA. In this region, TRPV4 has the highest homology with TRPV1 and TRPV2, with which it shares a central stretch of 11 highly conserved amino acids. There is also significant homology with TRPV5 and TRPV6 and, to a lesser extent, with the pore region of KcsA. When comparing
a possible involvement of pore residues in activation of TRPV4. In this manuscript, we focus on the contribution of these residues to typical pore properties: permeability, rectification, and voltage-dependent block.

Effects of Pore Mutations on Permeability to Mono- and Divalent Cations—It has been previously shown that TRPV4 is moderately selective for Ca\(^{2+}\)/H\(^{+}\) over Na\(^{+}\)/H\(^{+}\) ions and displays a monovalent cation permeability sequence corresponding to Eisenman IV. We investigated whether these properties are affected by mutations to the putative pore region by determining the relative cation permeabilities for all mutant channels. After full activation of the current with 4\(a\)-PDD, we switched between extracellular solutions containing a single permeant cation species and calculated relative permeabilities from the shifts in the reversal potential after correcting for liquid junction potentials (see “Materials and Methods”). For wild type TRPV4, switching from a Na\(^{+}\)-containing extracellular solution to solutions containing 30 mM Ca\(^{2+}\) as the sole charge carrier (arrow) in each panel, the resultant shift in reversal potential is indicated. The calculated values of \(P_{Ca}/P_{Na}\) are listed in Table I. G and H, leftward shift of the reversal potential for the M680D and M680D/ΔK675 mutants.

Equation 3 were 6.85 ± 0.55 (n = 25) and 2.52 ± 0.29 (n = 7) for Ca\(^{2+}\) and Mg\(^{2+}\), respectively. Substituting extracellular Na\(^{+}\) by other monovalent cations caused only small shifts in the reversal potential (Fig. 4A), confirming that TRPV4 poorly discriminates between monovalent cations. The deduced monovalent cation permeability sequence was K\(^{+}\) > Cs\(^{+}\) > Rb\(^{+}\) > Na\(^{+}\) > Li\(^{+}\), which corresponds to Eisenman sequence IV for a weak field strength site.

A possible explanation for the relatively low Ca\(^{2+}\) selectivity and the weak field strength of the TRPV4, TRPV1, and TRPV2 pores could be the presence of a positively charged lysine (Lys\(^{675}\) in TRPV4) in the central part of the putative pore region, which is not present in the strong field strength site TRPV5 and TRPV6 pores. However, neutralizing this lysine into alanine (mutant K675A) had no significant effect on the divalent selectivity of the channel (Fig. 3B). Likewise, the K675A pore still poorly discriminates between monovalent cations (Fig. 4B) and retains the Eisenman IV permeation profile. Thus, the low field strength binding site is conserved, indicating that Lys\(^{675}\) does not significantly influence the electrical field within the TRPV4 channel pore.

The two negatively charged residues in the pore region,
Asp<sup>672</sup> and Asp<sup>682</sup>, could potentially function as (part of) Ca<sup>2+</sup>-binding sites within the TRPV4 pore, similar to the role played by aspartate and glutamate residues in the Ca<sup>2+</sup>-selective pores of TRPV5, TRPV6, and voltage-gated Ca<sup>2+</sup> channels. Consistent with such a role, we found that the D672A and D682A mutants both displayed a reduced Ca<sup>2+</sup> selectivity, as can be judged from the less positive reversal potentials upon switching to a Ca<sup>2+</sup>-containing extracellular solution (Fig. 3, C and D). The relative Ca<sup>2+</sup> permeability (P<sub>Ca</sub>/P<sub>Na</sub>) was approximately halved in both mutants, to 3.20 ± 0.70 (n = 4; p < 0.05) and 4.09 ± 0.22 (n = 5; p < 0.05) for D672A and D682A, respectively (Table I). Likewise, inward current densities with 30 mM Ca<sup>2+</sup> as the sole charge carrier were significantly smaller for both mutants than for wild type TRPV4 despite the significantly larger current densities in Na<sup>+</sup>-containing solutions (Fig. 2), indicating that Ca<sup>2+</sup> conduction is hindered when these pore aspartates are neutralized. Like wild type TRPV4, the D672A and D682A pores discriminate poorly between monovalent cations and display the Eisenman IV permeation profile (Fig. 4C and data not shown). However, we found that both channels display a significantly higher relative permeability for K<sup>+</sup> ions, with P<sub>Ca</sub>/P<sub>Na</sub> values of 1.63 ± 0.10 (D672A, n = 5; p < 0.05) and 1.58 ± 0.07 (D682A, n = 6; p < 0.05) compared with 1.35 ± 0.03 (n = 10) for wild type TRPV4 (Table I).

Simultaneous neutralization of both aspartates (double mutant D672A/D682A) induced some more drastic changes in permeation phenotype. The relative Ca<sup>2+</sup> permeability was even more reduced than in the single mutants (Fig. 3E; P<sub>Ca</sub>/P<sub>Na</sub> = 2.45 ± 0.19, n = 12; p < 0.01), and there was no longer a measurable permeability for Mg<sup>2+</sup>. Moreover, the permeability sequence for monovalent cations was different from that of wild type, with both Cs<sup>+</sup> and Rb<sup>+</sup> being more permeant than K<sup>+</sup> in 7 of 8 cells (Fig. 4D). The observed Cs<sup>+</sup> > Rb<sup>+</sup> > K<sup>+</sup> > Na<sup>+</sup> > Li<sup>+</sup> permeability sequence is consistent with a reduction of the field strength of the cation-binding site to Eisenman sequence II or I (Table I). We also observed important differences in the rectification properties between wild type TRPV4 and the D672A/D682A mutant, which, as will be discussed below, arise from differences in Ca<sup>2+</sup> block.

From the sequence comparison in Fig. 1, two lines of evidence indicated that Met<sup>680</sup> is positioned at a potentially crucial position within the putative pore region of TRPV4. Firstly, at the corresponding position in TRPV5 we found an aspartate (residue 542), which is crucial for the high Ca<sup>2+</sup> selectivity of this channel. Secondly, at the corresponding position in voltage-gated K<sup>+</sup> channels there is a tyrosine, part of the so-called K<sup>+</sup> channel “signature sequence” (T(V/I)GYG), whose backbone atoms contribute to the lining of the K<sup>+</sup> selectivity filter. Mutants in which Met<sup>680</sup> was substituted by alanine or aspartate gave rise to sizable but strongly reduced current densities upon stimulation with 4α-PDD (Fig. 2), which may have some quantitative bearings on the determination of relative permeabilities of the M680A, M680D, and M680D/ΔK675 mutants. Still, some drastic changes in divalent permeability of these mutants were obvious. In the M680A mutant, switching from a Na<sup>+</sup>-containing extracellular solution a solution containing 30 mM Ca<sup>2+</sup> as the sole permeant caused a rightward shift of the reversal potential (Fig. 3F), corresponding to a P<sub>Ca</sub>/P<sub>Na</sub> value of 3.26 ± 0.09 (n = 6; p < 0.05). Under the same conditions, we observed a leftward shift of the reversal potential for the M680D and M680D/ΔK675 mutants (Fig. 3, G and H), indicating that these pore mutations abolish the Ca<sup>2+</sup> selectivity of the TRPV4 pore (P<sub>Ca</sub>/P<sub>Na</sub> values <1; p < 0.01). Compared with wild type TRPV4, no changes in the monovalent permeability sequence (Eisenman IV) were observed (data not shown), but all three mutants had a significantly higher relative permeability for K<sup>+</sup> (Table I).

Effects of Pore Mutations on the TRPV4 Rectification Properties—The current-voltage relation of wild type TRPV4 in extracellular solutions containing “physiological” concentrations of mono- and divalent cations displays both inward and outward rectification. As illustrated in Fig. 5A, this behavior strongly depends on the extracellular Ca<sup>2+</sup> concentration. In the absence of divalent cations, the current-voltage relation is linear with identical current amplitudes at +100 and −100 mV. Increasing concentrations of Ca<sup>2+</sup> cause an inhibition of the current that is more pronounced at negative potentials, characteristic of a voltage-dependent block. However, Ca<sup>2+</sup> concentrations up to 30 mM do not cause a complete block of the inward current, because Ca<sup>2+</sup> itself permeates the channel, as evidenced by the rightward shift of the reversal potential. To quantify the effect of extracellular Ca<sup>2+</sup> we normalized the inward current at −100 mV to the outward current at +100 mV, assuming that the Ca<sup>2+</sup> block disappears at highly positive potentials (Fig. 5D). For wild type TRPV4, the I<sub>-100 mV/I<sub>+100 mV</sub> ratio decreased from ~1 in nominally Ca<sup>2+</sup>-free solution to ~0.25 in the presence of 30 mM Ca<sup>2+</sup>. Virtually identical results were obtained for the K675A mutant, again indicating that this residue is not an important determinant of TRPV4 pore properties (Fig. 5D and data not shown). In contrast, neutralization of Asp<sup>672</sup> and Asp<sup>682</sup> strongly reduced the sensitivity of the channel to extracellular Ca<sup>2+</sup>. In the D672A/D682A double mutant, the current-voltage relation was still completely linear with 1 mM Ca<sup>2+</sup> in the extracellular solution.
extracellular RR is a potent, voltage-dependent blocker of wild type TRPV4. At a concentration of 1 μM, extracellular RR completely inhibited inward currents, whereas significant outward currents were measured at positive potentials more positive than +20 mV (Fig. 6, A and B). The apparent fraction of unblocked channels, measured from tail currents, increased from <0.05 at −100 mV to >0.8 at +80 mV, with 50% block at +60.8 ± 0.8 mV (n = 4; Fig. 6E). The rate of RR block was relatively fast, with a time constant for block of 0.9 ± 0.1 ms at −100 mV (n = 4). We also performed experiments in which a high concentration (50 μM) of RR was included in the pipette solution. Under this condition, we did not observe inhibition of the TRPV4 current, whereas a subsequent addition of 1 μM RR to the extracellular medium resulted in the expected voltage-dependent block. These data establish that RR blocks TRPV4 from the extracellular side by binding in a voltage-dependent manner to a site in the channel pore, within the transmembrane electrical field. Virtually identical results were obtained for the D672A and K680A mutants (Fig. 6E and data not shown). In contrast, the D682A and D672A/D682A mutants were much less sensitive to block by extracellular RR (Fig. 6, C and D, and data not shown), and the voltage dependence of the block was shifted to more negative potentials (Fig. 6E). For both mutants, the apparent fraction of unblocked channels increased from −0.15 at −100 mV to −1 at potentials >+40 mV (Fig. 6E), with 50% block at −21.0 ± 1.4 mV (n = 4) and −23.5 ± 2.5 mV (n = 3) for D672A/D682A and D682A, respectively. The rate of RR block was also much slower for these mutants, with time constants of 25.4 ± 1.3 ms (n = 4, D672A/D682A) and 24.7 ± 2.5 ms (n = 3, D682A). Thus, the negative side chain of Asp⁶⁶² appears to be crucial for the high sensitivity of TRPV4 for RR, similar to Asp⁶⁶⁶ in the pore of TRPV1 (18).

### DISCUSSION

In recent years, the persistent discovery of new members within the TRP superfamily of cation channels has greatly advanced our understanding of Ca²⁺ influx mechanisms in many cell types. However, it is not always clear whether the ionic currents that are observed after expression of members of the TRP family are directly mediated by the TRP proteins themselves or whether they merely act as modulators of endogenous channels. The situation is further complicated by the findings that TRP proteins can form heteromultimers (19) and/or reside in intracellular membranes (20). The analysis of channel permeation properties and modulation of these properties by mutations to pore residues form the ultimate proof of the channel nature of these proteins. At present, this goal has only been achieved for TRPV1 and TRPV5 (11, 18, 21). The present study adds another member of the TRPV family to this list, the osmosensitive and ligand-gated TRPV4. We have demonstrated that two negatively charged aspartate residues and

### TABLE I

| Channel   | \(P_{\text{Ca}}/P_{\text{Na}}\) | \(P_{\text{Mg}}/P_{\text{Na}}\) | \(P_{\text{K}}/P_{\text{Na}}\) | \(P_{\text{Mg}}/P_{\text{K}}\) | \(P_{\text{Ca}}/P_{\text{K}}\) | Eisenmann⁹⁰⁰¹ |
|-----------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|----------------|
| Wild type | 6.9 ± 0.6                     | 2.5 ± 0.3                     | 1.35 ± 0.03                   | 1.27 ± 0.04                   | 1.32 ± 0.03                   | 0.78 ± 0.08 IV (9/10) |
| K675A     | 6.3 ± 0.6                     | 1.7 ± 0.2                     | 1.35 ± 0.02                   | 1.32 ± 0.02                   | 1.26 ± 0.11                   | 0.73 ± 0.06 IV (17/21) |
| D672A     | 3.2 ± 0.7                     | 1.9 ± 0.2                     | 1.60 ± 0.14                   | 1.37 ± 0.02                   | 1.37 ± 0.16                   | 0.80 ± 0.09 IV (5/6)     |
| D682A     | 4.1 ± 0.3                     | 1.7 ± 0.2                     | 1.58 ± 0.07                   | 1.52 ± 0.04                   | 1.43 ± 0.09                   | 0.86 ± 0.06 IV (6/6)     |
| D672A/D682A | 2.5 ± 0.2                   | <1⁹⁰⁰²                        | 1.22 ± 0.03                   | 1.28 ± 0.03                   | 1.23 ± 0.02                   | 0.73 ± 0.03 I (5/8), II (2/8) |
| M680A     | 3.3 ± 0.1                     | 1.7 ± 0.1                     | 2.37 ± 0.34                   | 1.68 ± 0.11                   | 1.56 ± 0.05                   | 0.69 ± 0.10 IV (7/7)     |
| M680D     | <1⁹⁰⁰²                        | <1⁹⁰⁰²                        | 2.09 ± 0.15                   | 2.01 ± 0.30                   | 1.40 ± 0.09                   | 0.63 ± 0.06 IV (8/8)     |
| M680D/ΔK675 | <1⁹⁰⁰²                        | <1⁹⁰⁰²                        | 1.92 ± 0.09                   | 1.81 ± 0.26                   | 1.49 ± 0.09                   | 0.61 ± 0.07 IV (5/5)     |

⁹⁰⁰¹ The values in brackets indicate the number of times a selectivity sequence was observed and the number of trials.
⁹⁰⁰² Very small inward currents and strong leftward shift of the reversal potential, indicative of a negligible permeability.

### FIG. 5. Ca²⁺-dependent rectification of wild type and mutant TRPV4.

A, current-voltage relations for wild type TRPV4 in standard extracellular solution containing the indicated Ca²⁺ concentrations. Note the appearance of inward and outward rectification in the presence of Ca²⁺. B, same experiment as in A for the D672A/D682A double mutant. C, current voltage relations for wild type TRPV4 and three pore mutants measured with 5 mM extracellular Ca²⁺. The currents were normalized to the current at −100 mV. D, Ca²⁺ dependence of rectification properties for wild type and mutant TRPV4. Shown are the average ratios of the currents measured at −100 and +100 mV.

(Fig. 5B), and outward rectification remained reduced for Ca²⁺ concentrations up to 30 mM (Fig. 5, B and D). The Ca²⁺-dependent rectification properties of the single mutants D672A and D682A were intermediate between wild type and the D672A/D682A double mutant (Fig. 5D), which can also be appreciated from the normalized current-voltage relations measured with 5 mM extracellular Ca²⁺ (Fig. 5C). These results indicate that both aspartate residues contribute to the Ca²⁺ sensitivity of the TRPV4 pore.

### Effects of Pore Mutations on Ruthenium Red Sensitivity

Ruthenium Red (RR) is a polycationic molecule that has been shown to inhibit members of the TRPV family with nanomolar to low micromolar affinity. As illustrated in Fig. 6 (A and B),

The relative permeabilities were calculated as described under "Materials and Methods." The data are given as the means ± S.E. from at least three cells.

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Pore Mutations in TRPV4

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the non-polar amino acid methionine are critical determinants of the pore properties of TRPV4.

In the presence of both monovalent and divalent cations in the extracellular solution, the current-voltage relation of TRPV4 displays both inward and outward rectification, a feature it shares with TRPV1, TRPV2, and several members of the TRPM and TRPC families (22). Our present results show that, at least for TRPV4, this behavior depends on extracellular Ca\(^{2+}\) ions, which both block the channel in a voltage-dependent manner and permeate the channel. We found that two negatively charged residues, Asp\(^{672}\) and Asp\(^{682}\), contribute to the channel’s Ca\(^{2+}\) sensitivity, as evidenced by the reduced Ca\(^{2+}\)-dependent rectification and ∼2-fold lower relative Ca\(^{2+}\) permeability in both the D672A and D682A mutants. The D672A/D682A double mutant displays an even stronger reduction in Ca\(^{2+}\)-dependent rectification and Ca\(^{2+}\) permeability, indicating that the effect of neutralizing these aspartates on the Ca\(^{2+}\) sensitivity of TRPV4 is additive. Moreover, this double mutant has a very low selectivity for monovalent cations and a Cs\(^{+}\)/Rb\(^{+}\) selectivity of TRPV4 is additive. Moreover, this double mutant has a very low selectivity for monovalent cations and a Cs\(^{+}\)/Rb\(^{+}\) selectivity of 150 mM Na\(^{+}\) in the extracellular solution. Asp\(^{682}\) is likely to be located more toward the intracellular side of the channel, because it can interact with the permeant cation Ca\(^{2+}\) but not with the bulky, impermeanter cation Rb\(^{+}\).

Our results also indicate that Met\(^{680}\) is crucial for the proper functioning of the TRPV4 pore. Substituting this residue with an alanine results in a decreased Ca\(^{2+}\) permeability and overall current amplitude. Introducing a negative charge at this position (mutant M680D) causes a further reduction of the current density and a complete loss of Ca\(^{2+}\) permeation. One possible explanation is that this additional negative charge enhances the affinity of the Ca\(^{2+}\)-binding site to such an extent that it can no longer permeate the channel. We had hypothesized that neutralization of the positively charged lysine residue Lys\(^{675}\) could be involved in determining the field strength of the putative cation-binding site within the pore. This hypothesis originated from the fact that this residue is not present in the highly Ca\(^{2+}\)-selective channels TRPV5 and 6. However, permeation properties for monovalent cations are not altered, and the relative Ca\(^{2+}\) permeability remains unchanged in the K675A mutant. Likewise, the two other mutants that were constructed to transfer the high Ca\(^{2+}\) selectivity and strong field binding site of TRPV5/6 onto TRPV4 (M680D and M680D/D675) did not give the expected result and yielded channels with strongly reduced Ca\(^{2+}\) selectivity. It thus appears that the differences in pore properties between TRPV1/2/4 and TRPV5/6 cannot be reduced to single amino acid substitutions within the pore region.

As shown in Fig. 1, the pore region of TRPV4 shows significant homology with the pore region of KcsA, the bacterial K\(^{+}\) channel whose crystal structure has been determined at 2.0 Å resolution (23, 24). This raises the possibility that both channels share a similar pore architecture. The results from our mutagenesis study are at least consistent with this hypothesis. Firstly, as discussed above, we concluded that Asp\(^{682}\) is located at the extracellular part of the TRPV4 pore where it can interact with RR, whereas Asp\(^{672}\) seems to be located more toward the cytoplasm. Indeed, in the KcsA structure, Asp\(^{682}\) (corresponding to Asp\(^{682}\) in TRPV4) is located at the outer mouth of the selectivity filter, whereas Glu\(^{121}\) (corresponding to Asp\(^{672}\)) is directed more toward the intracellular side of the channel. Secondly, we found that Met\(^{680}\) is crucial for proper channel function, because mutating it to an alanine or aspartate strongly affects the permeability for monovalent and divalent cations. In the KcsA structure, the corresponding residue, Tyr\(^{78}\), is at the center of the selectivity filter, where its backbone oxygens interact with dehydrated K\(^{+}\) ions (23, 24). Finally, we concluded that the positively charged Lys\(^{675}\) does not significantly influence the pore properties of TRPV4. Interestingly, KcsA, like TRPV5/6, lacks a positive charge at the corresponding residue. However, if the structure homology between KcsA and TRPV4 holds true, Lys\(^{675}\) is expected to be part of the so-called pore helix (24), the residues of which do not directly contribute to the lining of the channel pore.
basic pore architecture as K+ channels, although further work, including a determination of the multimeric structure of the channel, may be needed to substantiate this notion. The present work may also form a starting point for future studies aimed at the unraveling of the exact gating mechanism or the discovery of more selective TRPV4 antagonists.

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