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Calcium-dependent decrease in the single channel conductance of TRPV1

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Abstract

TRPV1 is a Ca^{2+} permeable cation channel gated by multiple stimuli including noxious heat, capsaicin, protons, and extracellular cations. In this paper, we show that Ca^{2+} causes a concentration and voltage-dependent decrease in the capsaicin-gated TRPV1 single channel conductance. This Ca^{2+} -dependent effect on conductance was strongest at membrane potentials between -60 mV and $+20$ mV, but was diminished at more hyperpolarised potentials. Using simultaneous recordings of membrane current and fura-2 fluorescence to measure the fractional Ca^{2+} current of whole cell currents evoked through wild type and mutant TRPV1 receptor, we investigated a possible link between the mechanisms underlying Ca^{2+} permeation and the Ca^{2+} -dependent effect on conductance. Surprisingly we found no evidence of a structural correlation, and observed that the substitution of amino acids known to regulate Ca^{2+} permeability had little effect on the ability for Ca^{2+} to decrease TRPV1 conductance. However, we did observe that the Ca^{2+} -dependent effect on conductance was not diminished by negative hyperpolarization for a mutant receptor with severely impaired Ca^{2+} permeability, TRPV1-D646N/E648Q/E651Q. This would be consistent with the idea that Ca^{2+} reduces conductance by interacting with an intra-pore binding site, and that negative hyperpolarization reduces occupancy of this site by speeding the exit of Ca^{2+} into the cell. Taken together, our data show that in addition to directly and indirectly regulating channel gating, Ca^{2+} also directly reduces the conductance of TRPV1. Surprisingly, the mechanism underlying this Ca^{2+} -dependent effect on conductance is largely independent of mechanisms governing Ca^{2+} permeability.

Keywords

vanilloid receptor; capsaicin; ligand-gated ion channel; Ca^{2+} permeability; TRP channels

Introduction

TRPV1 is one of four thermosensitive members of the vanilloid receptor subclass of *Transient receptor potential (Trp)* family of ion channels, and is of particular interest because of its involvement in pain sensation [1, 2]. It displays marked polymodality and is activated by a diverse range of stimuli including capsaicin, protons, high temperature, ethanol, anandamide, polyamines, (N-vanillyl)-9-oleamide, spider venom toxins, and elevated concentrations of extracellular polyvalent cations including divalent cations [3–10].

Calcium plays a key role in TRPV1 function. At low millimolar concentrations, Ca^{2+} and other divalent cations sensitize TRPV1 to gating by thermal and chemical stimuli, whereas

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at higher concentrations Ca^{2+} directly activates the channel [4]. Further, TRPV1 carries a significant transmembrane Ca^{2+} current that both initiates and modulates a number of key downstream physiological and pathophysiological processes [11]. We recently showed that the fraction of the total agonist-gated current carried by Ca^{2+} , called the “fractional Ca^{2+} current” or $P_f\%$, is a tunable property that depends on the nature of the agonist used to activate the receptor [12]. The $P_f\%$ is smaller for TRPV1 currents evoked by protons (~6%) than for those evoked by capsaicin (~10%) because H^+ and Ca^{2+} compete for common binding sites composed of acidic side chains in the pore.

In the present study, we investigated the effect of Ca^{2+} on the single channel conductance of TRPV1 in outside-out membrane patches excised from transfected HEK293 cells. Ca^{2+} is known to reduce the monovalent cation currents of voltage-gated Na^+ [13, 14] and Ca^{2+} channels [15], glutamate-gated NR1/NR2B receptors [16], cyclic nucleotide-gated (CNG) channels [17], and ATP-gated P2X receptors [18]. In some cases, these effects result from a direct block of current as Ca^{2+} binds to sites in the permeation pathway [19]. Further, in Ca^{2+} permeable channels, Ca^{2+} selectivity and block often share common structural loci. For example, Ca^{2+} selection in voltage-gated Ca^{2+} channels [20] and CNG channels [17] is in large part attributable to high affinity binding sites in the channel pore that show a strong preference for divalent cations, thereby reducing monovalent cation conductance while facilitating divalent cation conductance. In a similar fashion, Ca^{2+} binding to a locus in the extracellular mouth of the NMDA channel pore results in a reduction in monovalent conductance and a facilitation of Ca^{2+} conductance [16, 21]. Despite these examples, no studies to date report a reduction of TRPV1 current by Ca^{2+} , although previous studies have observed a modest reduction in unitary currents by Mg^{2+} and Ni^{2+} [4, 22].

In this paper we report that Ca^{2+} also causes a concentration- and voltage-dependent reduction in TRPV1 conductance, but that surprisingly the mechanism appears to be largely independent of that responsible for regulating Ca^{2+} permeability.

Materials and Methods

Molecular Biology and cell culture

Single amino acid substitutions were introduced into the rat wild-type TRPV1 cDNA background using the Quikchange Lightning Site-Directed Mutagenesis Kit (Stratagene) and verified by automated DNA sequencing (Retrogen).

Human embryonic kidney-293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin G, and 50 $\mu\text{g}/\text{ml}$ streptomycin (Invitrogen) at 37°C in a humidified, 5% CO_2 atmosphere. Cells were transfected with cDNAs encoding wild-type or mutant TRPV1 and a fluorescent reporter using Lipofectamine LTX (Invitrogen). For measurements of single channel currents and $P_f\%$, cells were re-plated onto poly-L-lysine coated glass coverslips (Gold Seal, Becton-Dickinson) the day before the experiment. For measurements of relative ionic permeability, we used round cells that were detached from culture dishes by mechanical dispersion using a physiological saline solution and a fire-polished Pasteur pipette.

Electrophysiology and drug application

Membrane currents were recorded with AxoPatch 200B amplifiers (Molecular Devices) using conventional broken-patch methods [23]. Data were digitized at 20 kHz with Instrutech ITC-16 acquisition hardware (HEKA Instruments) and AxoGraphX software (AxoGraph Scientific). The stored data were analysed off-line using AxoGraphX and Igor Pro software (WaveMetrics). We activated the channel using either capsaicin (0.1–1 μM) or a high (>10 mM) concentration of a polyvalent cation [4]. Capsaicin was applied using a

SF-77B Perfusion Fast-Step solution switcher (Warner Instruments), or by slow perfusion (~1 ml/min) through the bath's bulk solution inflow.

Single channel recordings—Single channel currents were measured in outside-out membrane patches using 6–10 MΩ borosilicate glass pipettes (Warner Glass) containing (in mM): 150 NaCl, 10 EGTA, 10 HEPES, and 4 NaOH (pH 7.4). Current was low-pass filtered at 5–10 kHz and digitized at 20 kHz before storage and analysis. All data traces presented in the figures were further processed using a 1 kHz low-pass software filter routine (Igor Filter Design Laboratory, WaveMetrics). Mean single channel current amplitude (i) was determined from the multi-Gaussian fit of the all points histogram a trace of single channel data of >10 s in length. measured at a range of membrane potentials. Single channel chord conductance (g) was calculated as follows:

$$g = \frac{i}{(E_m - E_{Rev})}, \quad (\text{Eq. 1})$$

where i is the single channel current amplitude, E_m is membrane potential, and E_{Rev} is the reversal potential of the agonist-gated current. We assumed that E_{Rev} equalled ~0 mV when the extracellular solution contained less than 10 mM added CaCl_2 . To calculate conductance in the presence of 10 mM extracellular CaCl_2 added to 154 mM NaCl, E_{Rev} was determined from whole cell current reversal potentials obtained under the same conditions and equalled 8.1 ± 0.76 mV ($n=8$). When 112 mM extracellular CaCl_2 was present with 154 mM intracellular NaCl, E_{Rev} was 24.8 ± 1.2 mV ($n=13$). For 112 mM extracellular Mg^{2+} , it was 15.8 ± 1.4 mV ($n=4$). The relative ionic conductance sequence was determined from chord conductances measured in sequential extracellular solutions of a general composition of (mM): 150 X^+Cl or 110 Y^{2+}Cl_2 , 10 glucose, 10 HEPES, and 4 X^+OH or 2 $\text{Y}^{2+}(\text{OH})_2$ (pH 7.4); where X^+ is Na^+ , K^+ , Li^+ , Cs^+ , and Y^{2+} is Ca^{2+} or Mg^{2+} . To measure the ability of Ca^{2+} to block monovalent cation current, Ca^{2+} (0.1–30 mM) was added to a solution containing (mM): 150 NaCl, 4 NaOH, 10 glucose, and 10 HEPES (pH 7.4). Indifferent electrodes were suspended in 3 M KCl agar bridges in contact with the bath solutions.

Measurements of relative permeability—Whole-cell current was recorded using low resistance (0.5–3 MΩ), borosilicate glass pipettes (World Precision Instruments) filled with (mM): 150 NaCl, 4 NaOH, 10 EGTA, and 10 HEPES (pH 7.3). The extracellular solutions were (mM): 150 X^+Cl or 110 Y^{2+}Cl_2 , 10 glucose, 10 HEPES, and 4 X^+OH or 2 $\text{Y}^{2+}(\text{OH})_2$ (pH 7.4). Indifferent electrodes were suspended in 3 M KCl agar bridges in contact with the bath solutions. Current-voltage curves were generated by changing the membrane voltage from –80 mV to +60 mV at a constant rate (1.4V/s) before and during applications of agonist. E_{Rev} was then measured by subtracting the evoked current ramp obtained in the presence of agonist from the leak current ramp obtained in the absence of agonist. For monovalent test cations, relative permeability was calculated using the following equation:

$$P_{\text{X}^+}/P_{\text{Na}^+} = e^{\{\Delta E_{Rev} F/RT\}} \quad (\text{Eq. 2})$$

where ΔE_{Rev} is the measured shift in reversal potential, and where R , T and F have their usual meanings and the value RT/F was 25 mV (20 °C). For divalent cations, the following equation was used [19]:

$$P_{\text{Y}^{2+}}/P_{\text{Na}^+} = \frac{\gamma_{\text{Na}^+} * [\text{Na}^+]_i}{4 * \gamma_{\text{Y}^{2+}} * [\text{Y}^{2+}]_o} * e^{\{\Delta E_{Rev} F/RT\}} * (e^{\{\Delta E_{Rev} F/RT\}} + 1) \quad (\text{Eq. 3})$$

where ΔE_{Rev} is the measured shift; γ_{Na^+} , $\gamma_{Y^{2+}}$ represent the activity coefficients for Na^+ (0.75 for 154 mM), and the test divalent cation (0.26 for 112 mM Ca^{2+} or Mg^{2+}) respectively.

Patch clamp photometry—The $Pf\%$ for whole-cell TRPV1-mediated current was determined as previously described [24]. Briefly, whole-cell currents were recorded from adherent HEK293 cells using recording electrodes containing (mM): 140 CsCl, 3 CsOH, 2 K_5 -fura-2, 10 tetraethylammonium, and 10 HEPES (pH 7.3). These cells were perfused with an extracellular solution containing (in mM): 140 NaCl, 4 NaOH, 0.3–10 $CaCl_2$, 10 glucose, and 10 HEPES (pH 7.4). Fura-2 fluorescence (380 nm excitation; 510 nm emission) was simultaneously measured with a Photomultiplier Detection System (Photon Technologies Inc). Then, $Pf\%$ was calculated as:

$$Pf\% = \frac{\Delta F_{380}/F_{max}}{Q_T} * 100, \quad (\text{Eq. 4})$$

where ΔF_{380} is the change in fura-2 fluorescence caused by Ca^{2+} entry, Q_T is the total integrated current recorded using patch clamp electrophysiology, and F_{max} is a proportionality determined in separate experiments (see [24]).

Statistical Tests and Reports—Mean current amplitude data are presented with $\pm$ S.E.M of at least three experiments. Comparison between two data sets was conducted using the Student's t -test. Groups of data were analyzed by one-way ANOVA with significance determined from the Tukey's protected multiple comparison test using Instat (GraphPad Software, Inc., La Jolla, CA), with $p < 0.05$ considered significant.

Results

Cation conductance and relative permeability

We recorded inward single TRPV1 channel currents from outside-out membrane patches held at -60 mV and bathed in a range of extracellular solutions in which the sole cationic charge carrier was either Na^+ , K^+ , Cs^+ , Li^+ , Ca^{2+} or Mg^{2+} . In all recordings, we detected both a single dominant main conductance state in addition to rare subconductance states [25]. The subconductance state occurred too infrequently to reliably characterize, and thus we focused on the main conductance state only.

We found that the single channel chord conductance of TRPV1 showed little dependence on the nature of the external monovalent charge carrier, ranging from a high of 62.8 ± 4.3 pS in the presence of extracellular Na^+ to a low of 53.1 ± 2.3 pS in K^+ (Fig. 1, Table 1). In contrast, we observed significantly ($p < 0.001$) smaller chord conductances in the presence of Ca^{2+} or Mg^{2+} (15.2 ± 0.7 pS and 14.6 ± 1.1 pS, respectively).

We then measured the permeability of each cation relative to Na^+ (P_X/P_{Na}) from whole-cell, bi-ionic reversal potential measurements and the Goldman-Hodgkin-Katz (GHK) constant field voltage equation [19] (Table 1). Consistent with published work [6], we found that TRPV1 poorly discriminates between monovalent cations ($P_X^+/P_{Na} \sim 1$), but shows a strong preference for divalent cations (P_{Ca}/P_{Na} and P_{Mg}/P_{Na} were 10.2 ± 0.8 and 5.5 ± 0.5 , respectively). Thus, although TRPV1 is more selective for Ca^{2+} and Mg^{2+} relative to monovalent species, these divalent cations have lower conductances. High selectivity and low conductance is consistent with the presence of a binding site within the permeation pathway that prefers divalent to monovalent cations. If this is the case, then the divalent cation selectivity is high because the site preferentially binds Ca^{2+} and Mg^{2+} , and divalent cation conductance is low because slow unbinding retards conduction through the pore [26].

Ca²⁺-dependent decrease in TRPV1 conductance

We next measured the effect of increasing concentrations (0.1–30 mM) of the Group 2 Metal Ions, Ba²⁺, Ca²⁺, and Mg²⁺, on TRPV1 single channel current of outside-out patches bathed in an extracellular solution containing 154 mM Na⁺. We observed a concentration-dependent decrease in single channel conductance with increasing concentrations of extracellular divalents (Fig. 2). Accurate determination of maximal conductance inhibition by divalent cations was limited due to the effects of adding increasingly higher extracellular divalent cation chloride salts on the osmotic potential across the plasma membrane. Indeed, patches became unstable when divalent cation concentrations exceeded 30 mM (likely due to osmotic stress). Nevertheless, we were able to estimate and compare IC₅₀ values from the concentration-response data for the divalent cation-dependent decrease in conductance. The IC₅₀ for Ba²⁺, Ca²⁺, and Mg²⁺ were 1.0, 2.3 and 8.4 mM, respectively (Fig. 2b, c and d). The difference in potency among divalents is clearly seen in the results obtained in the absence and presence of 2 mM of each cation (Supplemental Figure 1). At this concentration, the unitary conductance in the presence of Mg²⁺ (60.9 ± 3.1 pS, $n = 7$) was not significantly different from control (62.8 ± 4.3 pS, $n=25$) ($p>0.05$). In contrast, 2 mM Ca²⁺ reduced the unitary conductance to $42.7.1 \pm 1.1$ pS ($n = 20$; $p<0.001$), and 2 mM Ba²⁺ reduced the single channel conductance even further to 30.1 ± 3.0 pS ($n = 5$), which was significantly different from both control ($p<0.001$) and that measured in 2 mM Ca²⁺ ($p<0.01$).

We next looked at the voltage-sensitivity of the Ca²⁺-dependent decrease in channel conductance (Fig. 3). Unitary monovalent current amplitude showed outward rectification in the absence of divalent cations (Fig. 3c, black circles), which is consistent with previous reports [6, 9, 25, 27]. The chord conductance of TRPV1 at different [Ca²⁺]_o and membrane potential were calculated according to Equation 1 (see Supplemental Fig 2). The voltage-dependence of the Ca²⁺ inhibition of TRPV21 conductance by Ca²⁺ can then be seen more clearly in Fig. 3d, where chord conductances are normalized to the respective control measurements made in nominally free [Ca²⁺]_o. The Ca²⁺-dependent decrease in conductance was most pronounced at membrane potentials between –60 and +20 mV, but was diminished beyond this range. For example, the conductance measured at –80 mV and –100 mV in the absence of added [Ca²⁺]_o were 50.1 ± 3.8 pS ($n = 8$) and 55.5 ± 8.2 ($n = 5$) respectively, which are not significantly different from those measured in 2 mM [Ca²⁺]_o (49.6 ± 2.0 pS, $n = 7$, and 48 ± 7.1 pS, $n = 4$, respectively).

Taken together, the data show that divalent cations reduce TRPV1 conductance in a concentration- and voltage-dependent manner, and suggest that TRPV1 experiences a tonic depression of monovalent current at physiological concentrations of [Ca²⁺]_o and at normal resting membrane potentials.

Ca²⁺- and voltage-dependence of Pf%

We continued to explore the hypothesis that a common structural locus influences both the permeation and blocking properties of cations by asking the following question: Is Ca²⁺ current through the channel affected by Ca²⁺ occupying a blocking site within the pore? To address this issue, we first measured the effect of changing [Ca²⁺]_o on the fractional contribution of Ca²⁺ to the TRPPV1 current, or Pf%, at a fixed membrane potential of –60 mV (Fig. 4a). The Pf% has an advantage over traditional reversal potential-based methods because it allows Ca²⁺ permeability to be evaluated at a range of membrane potentials, including those within the range of membrane potentials experienced by a typical excitable cell [28]. We found that Pf% did not substantially deviate from values estimated using the GHK model as [Ca²⁺]_o was increased from 0.3 to 10 mM (Fig. 4b), the linear range of the concentration-effect curve over which Ca²⁺ decreased conductance. These data suggest that

Ca^{2+} permeability is not dependent on Ca^{2+} occupancy of the divalent cation blocking site as $Pf\%$ did not deviate substantially from the predictions of the GHK model. Next, we examined the relationship between $Pf\%$ and membrane potential. $Pf\%$ measurements were made in 2 mM $[\text{Ca}^{2+}]_o$ at membrane potentials in the range of -80 to -10 mV (Fig. 4c and d). Although 2 mM $[\text{Ca}^{2+}]_o$ did not cause a significant block of TRPV1 conductance at -80 mV (see Fig. 4c and d) the $Pf\%$ measured at this membrane potential and Ca^{2+} concentration was not significantly different from that recorded at -60 mV (Fig. 3c), suggesting again that selection and block are not closely tied. The empirically-derived $Pf\%$ was observed to deviate from the GHK model prediction at membrane potentials more positive than -60 mV (Fig. 4d). However, the ability for Ca^{2+} to reduce conductance was not greatly affected over this voltage range (Fig. 3d), which also provides little support the idea that Ca^{2+} permeability is correlated with Ca^{2+} occupancy of the blocking site. Rather, the observed deviation may be a consequence of the outwardly rectifying nature of the monovalent current through TRPV1, which implies enhanced outward monovalent conductance as membrane potential approaches zero. This would have the effect of reducing the net inward current and cause the $Pf\%$ to appear higher even though Ca^{2+} influx relative to Na^+ influx might not be changed. Further experiments would be required to confirm this hypothesis.

Taken together, these data show little evidence for a correlation between Ca^{2+} permeability relative to Na^+ and the probability that Ca^{2+} is occupying the binding site(s) responsible for reducing TRPV1 conductance. To explore this more directly, we next investigated whether mutation of amino acids known to regulate Ca^{2+} selection and permeability might alter the ability of Ca^{2+} to reduce TRPV1 conductance.

Effect of site-directed mutagenesis on the Ca^{2+} -dependent reduction in TRPV1 conductance

In many ion channels the negatively charged side chains of aspartate and glutamate interact with Ca^{2+} [19]. Although no accurate 3-D map of the TRPV1 receptor exists, the channel is thought to share structural homology with voltage-gated K^+ channels [29]. If this is so, then each of the four subunits of the TRPV1 tetramer contains four negatively charged amino acids located in or near the putative selectivity filter. Glu⁶³⁶ resides in the putative pore helix of the channel, whereas Asp⁶⁴⁶, Glu⁶⁴⁸ and Glu⁶⁵¹ are perched on the top of the selectivity filter (Fig. 5a). Previously, we showed that individual substitution of Asp⁶⁴⁶, Glu⁶⁴⁸ and Glu⁶⁵¹ with neutral residues significantly attenuated Ca^{2+} flux [12]. Negatively charged side chains could influence the divalent cation-mediated reduction of current in two ways. First, they may produce a local surface potential capable of concentrating Na^+ in the outer mouth of the channel pore, thus enhancing its inward conductance. Divalent cations are known to screen surface charge on membranes and protein [30], and thus increasing $[\text{Ca}^{2+}]_o$ would lower the ability of the local electrostatic potentials to attract Na^+ ions into the TRPV1 pore. Second, Na^+ and Ca^{2+} might compete for a common binding site formed by the acidic side chains. If the affinity of the site is higher for Ca^{2+} than it is for Na^+ , then increasing $[\text{Ca}^{2+}]_o$ would block Na^+ current in a concentration-dependent manner.

In a recent paper, Liu and co-workers showed that neutralizing the side chain charge on both Glu⁶³⁶ and Asp⁶⁴⁶ [31] abolished proton-dependent inhibition of TRPV1 conductance. We first investigated whether Glu⁶³⁶ influenced Ca^{2+} flux by substituting it with a neutral Gln residue and measuring the resulting receptor's $Pf\%$. Consistent with a previous work utilizing a reversal potential-based approach [32], we found that the TRPV1-E636Q mutant exhibited a significantly increased $Pf\%$ of $18.2 \pm 0.7\%$ ($n=3$). In contrast, the $Pf\%$ of the double mutant, TRPV1-E636Q/D646N (TRPV1-QN), was significantly lower ($4.4 \pm 0.7\%$, $n=3$) than that of the WT receptor, and approximately equal to that previously reported for the TRPV1-D646Q single mutant [12]. Despite having a moderate effect on Ca^{2+} flux,

however, 2 and 10 mM $[Ca^{2+}]_o$ decreased the current amplitude of TRPV1-QN to the same degree as observed for the wild-type receptor (Fig. 5b,c;n=4-10).

We have previously shown that individual substitution of Asp⁶⁴⁶, Glu⁶⁴⁸ or Glu⁶⁵¹ attenuated TRPV1 *Pf*%. Nevertheless, substitution of these residues for neutral counterparts, Asn, Gln and Gln respectively, did not significantly alter the effect of a half-maximally effective $[Ca^{2+}]_o$ on TRPV1 conductance (Fig. 5c). We went further and generated a mutant with neutral residues introduced at all three of these side chains, D646N/E648Q/E651Q (TRPV1-NQQ). Again, at -60 mV the conductance of this channel was similarly reduced by 2 and 10 mM $[Ca^{2+}]_o$ in a manner not significantly different from the wild-type receptor.

Consistent with our previous work implicating Asp⁶⁴⁶, Glu⁶⁴⁸ and Glu⁶⁵¹ in regulating Ca^{2+} permeability, we found that TRPV1-NQQ exhibited a profoundly reduced *Pf*%, with no measurable change in fura-2 fluorescence when the channel was activated in the presence of 2 mM $[Ca^{2+}]_o$. In cells expressing both the TRPV1-NQQ channel and the Ca^{2+} permeable ATP-gated P2X2 receptor [24], we measured clear changes in fura-2 fluorescence with application of ATP, but no change in fluorescence with capsaicin; thus, the lack of a measurable *Pf*% for TRPV1-NQQ was not an artefact of the system due to fura-2 saturation or leak (Fig. 6). We then increased extracellular $[Ca^{2+}]_o$ to 10 mM, after which we saw a clear deflection in fura-2 fluorescence in response to capsaicin (10 μ M); the *Pf*% measured in this way was $12.3 \pm 0.8\%$ (n=3). Given that the relationship between $[Ca^{2+}]_o$ and Ca^{2+} permeability is reasonably well described by the constant field model (see Fig. 4b), we estimated P_{Ca}/P_{Na} to be 1.1 ± 0.09 (n=3) based on the following form of the Lewis equation [33]:

$$\frac{P_{Ca}}{P_{Na}} = \frac{\gamma_{Na} * [Na]_o - (P_{Cs}/P_{Na} * \gamma_{Cs} * [Cs]_i * e^{(2E_{rev}F/RT)})}{(4 * \gamma_{Ca} * [Ca]_o * (1/Pf\% - 1))},$$

where the activity coefficients were as follows: γ_{Ca} , 0.3; γ_{Cs} and γ_{Na} , 0.75. The value 0.94 was used for P_{Cs}/P_{Na} (see Table 1). These results imply that neutralizing all three charged side chains at positions 646, 648 and 651 virtually removes all preference TRPV1 has for Ca^{2+} relative to Na^+ .

We next tested the hypothesis that the reduced potency with which Ca^{2+} depressed TRPV1 conductance at hyperpolarized potentials was due to a “push through” effect. This would destabilize the interaction between Ca^{2+} and its putative pore binding site and facilitate Ca^{2+} entry into the cell. If the hypothesis was accurate, we would expect hyperpolarization to have less of an effect on the Ca^{2+} -dependent inhibition of TRPV1-NQQ conductance due to the significantly retarded ability for Ca^{2+} to permeate thoroughly through the channel of this receptor. Fig 7 compares the voltage-current curves at a range of negative holding potentials for wild-type and TRPV1-NQQ receptors in the absence of divalent cations or the presence of 10 mM $[Ca^{2+}]_o$. Although the mutants exhibit similar current amplitudes in the presence of 10 mM $[Ca^{2+}]_o$ between -40 and -80 mV, at potentials more negative than -80 mV the wild-type receptor current begins to inwardly rectify and converge with the current amplitude values obtained in divalent cation free solution. In contrast, the current voltage profile in 10 mM $[Ca^{2+}]_o$ for TRPV1-NQQ does not inwardly rectify, even at potentials as negative as -120 mV. This implies that the Ca^{2+} -dependent decrease in TRPV1-NQQ conductance is not relieved by hyperpolarization in this mutant, a result that is consistent with the hypothesis that the site conferring the Ca^{2+} -dependent effects on conductance is within the permeation pathway.

Taken together, these data provide further evidence that the structural locus at which divalent cations interact and cause a reduction in TRPV1 conductance is separate from that regulating Ca^{2+} permeability, but that it is still likely to be within the permeation pathway.

Discussion

Ca^{2+} plays multiple roles in TRPV1 channel function. It modulates channel gating, directly activates the channel, and carries a significant percentage of the TRPV1 current. Here we report of novel (but not unexpected) effect of Ca^{2+} on TRPV1 channel function: that is, we find that divalent cations reduce TRPV1 conductance in a manner consistent with an interaction with a binding site within the electrical field of the permeation pathway. This effect is readily apparent at physiological membrane potentials and at the low millimolar concentrations of Ca^{2+} that occur naturally. Thus, Ca^{2+} , and to a lesser extent Mg^{2+} , cause a tonic inhibition of TRPV1 channel function.

Although divalent cations affect the conductance of many types of ion channels by shielding surface charge [30], this does not seem to be the case for TRPV1, for two reasons. First, we found that the reduction in TRPV1 current depended on the divalent cation species, which is inconsistent with simple charge screening models that do not discriminate between ions of like charge [30]. Second, neutralization of acidic side chains in the mouth of TRPV1 pore had no effect on the Ca^{2+} -dependent reduction in conductance caused by a half-maximally effective $[\text{Ca}^{2+}]_o$.

A second possibility, consistent with the voltage-dependence of the block, is that Ca^{2+} interacts with one or more binding sites within the permeation pathway of the channel, and thus reduces cation current by a steric occlusion or electrostatic repulsion. However, both TRPV1 conductance and gating are known to be voltage-dependent [25, 34], and it's conceivable that Ca^{2+} could affect conductance by modulating a voltage-sensor that resides in a part of the protein that is within the plane of the membrane but not necessarily within the channel pore. A piece of evidence in support of the Ca^{2+} binding site being within the pore is that hyperpolarization relieves the Ca^{2+} -dependent decrease in conductance in the wild-type TRPV1 receptor, which is expected if the increased electromotive force facilitates "push through" of Ca^{2+} from an intra-pore binding site. The Ca^{2+} -dependent conductance decrease of mutant receptor in which Ca^{2+} permeation is substantially retarded, TRPV1-NQQ, is less affected by hyperpolarization, which is likely because a commensurately greater driving force is required for pore-bound Ca^{2+} to overcome the energy barrier for permeation into the cell.

Highly selective Ca^{2+} permeable channels often show single-file conduction where the presence of one permeable ion in the channel influences the conduction of a second (i.e., single-file, multi-ion block) [20]. In voltage-gated Ca^{2+} channels this is revealed by an "anomalous mole fraction effect" in mixed divalent cation solutions, and by the observation of a biphasic conductance profile when titrating a fixed external $[\text{Na}^+]_o$ solution with increasing $[\text{Ca}^{2+}]_o$ [15]. However, within the concentration ranges used in this paper, we saw no evidence of the biphasic concentration-effect profile encountered when ions bind to multiple sites in the pore with different affinities. A second consequence of a single-file, multi-ion scheme is that one would predict a concentration-dependence of Ca^{2+} permeability, with $Pf\%$ approaching 100% as the probability of Ca^{2+} occupying the pore approached unity [35]. In our studies, the concentration-dependence of the $Pf\%$ of TRPV1 actually followed fairly closely to that predicted by simple constant field theory, suggesting that $P_{\text{Ca}}/P_{\text{Na}}$ is independent of the likelihood that Ca^{2+} is occupying the site responsible for decreasing conductance. In short, we find no compelling evidence for a multi-ion pore model that would describe the complex relationship between Ca^{2+} and the TRPV1 pore.

What is the structural locus mediating the effects of divalent cations on conductance?

We initially assumed that divalent cations reduced TRPV1 conductance by interacting with the same residues previously implicated in regulating the proton-mediated decrease in conductance [31]. However, we were surprised to find that the conductance of the double mutant, TRPV1-QN, was still reduced by Ca^{2+} even though pH was shown to have little effect.

Our subsequent hypothesis was that the divalent cation-dependent reduction in conductance was directly related to ion-pore interactions underlying ion selection and permeation, as is the case for other Ca^{2+} permeable channels. Neutralizing the charge on the three acidic side chains, Asp⁶⁴⁶, Glu⁶⁴⁸, and Glu⁶⁵¹ has been previously shown to reduce Ca^{2+} permeability in TRPV1 using both reversal potential measurements and measurements of *Pf*% [12, 32, 36, 37]. The role of these side chains in regulating permeation was further confirmed in this paper, where we show that the *Pf*% of the triple mutant, TRPV1-NQQ, was only measurable in the presence of elevated $[\text{Ca}^{2+}]_o$ and demonstrated an almost complete loss of selectivity between Na^+ and Ca^{2+} ions. Note that this is not necessarily evidence that Asp⁶⁴⁶, Glu⁶⁴⁸ and Glu⁶⁵¹ constitute the actual selectivity filter for divalent cations; it is also plausible that mutating these residue alters the structure of a selectivity filter located deeper in the channel pore, perhaps in the area analogous to the selectivity filter present in potassium channel family members. Nevertheless, we found that Ca^{2+} still effectively reduced conductance through TRPV1 mutants bearing substitutions at one or all of these positions.

Although our experiments did not succeed in locating the site responsible for mediating the Ca^{2+} dependent decrease in conductance, our data suggest that it likely lies within the channel pore, albeit at a locus distinct from that previously implicated in Ca^{2+} permeation [12, 32, 37, 38]. A more systematic approach involving a complete scan of the remaining non-acidic pore-lining residues within TRPV1 may yet reveal the distinct structural locus conferring the observed divalent cation-dependent reduction in conductance. However, it is also possible that the Ca^{2+} -dependent effects on conductance reported here do not involve amino acid side-chains *per se*, but rather results from an interaction with backbone carbonyl oxygens, as recently suggested by studies of the bacterial NaK channel [39, 40].

Concluding remarks

In this paper we show that extracellular Ca^{2+} reduced conductance through TRPV1 well within the physiological range of extracellular $[\text{Ca}^{2+}]_o$ and within the physiological range of membrane potentials experienced by excitable cells. Thus, Ca^{2+} can have competing effects on the total charge transported by TRPV1. On the one hand, elevating $[\text{Ca}^{2+}]_o$ up to 10 mM has a positive effect on the rate at which TRPV1 transports cations across the membrane by increasing the channel's open probability in the presence of agonists [4]. On the other hand, elevating $[\text{Ca}^{2+}]_o$ over this range suppresses the rate of cation transport by directly reducing TRPV1 conductance. A further negative effect of Ca^{2+} is exerted from the intracellular side, where it works to decrease open probability through a calmodulin-dependent process [41] that likely underlies the Ca^{2+} -dependent desensitisation of whole cell currents [42].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Single channel TRPV1 currents in presence of different extracellular cations
Representative single channel recordings (left) and associated amplitude frequency histograms (right, y-axis = frequency) of capsaicin-evoked currents in outside-out membrane patches excised from TRPV1-expressing HEK293. For monovalent cation currents, recordings were made in the presence of 154 mM extracellular Na^+ , Li^+ , K^+ or Cs^+ . For divalent cation currents, 112 mM extracellular Ca^{2+} , or Mg^{2+} was used. In all cases, the pipette contained 154 mM Na^+ . Closed and open channel states are indicated by the letters 'c' and 'o' respectively.

Figure 2. Divalent cation inhibition of TRPV1 conductance

a–c) Representative outside-out patch single channel recordings (left) and associated amplitude frequency histograms of TRPV1-mediated currents in differing concentrations of Ca^{2+} (**a**), Mg^{2+} (**b**), and Ba^{2+} (**c**). **(d)** Concentration-response relationship between normalized mean current amplitude and divalent cation concentration (filled black circles, Ca^{2+} ; blue, Mg^{2+} ; red, Ba^{2+} ; $n=3-20$). Curves were fitted using the Hill equation giving estimated IC_{50} values of 2.3, 1.0 and 8.4 mM for Ca^{2+} , Ba^{2+} and Mg^{2+} respectively. The mean amplitude of single channel currents evoked in the presence of a Na^+ -free extracellular solution containing 112 mM Ca^{2+} or 112 mM Mg^{2+} are shown for reference (open circle and triangle respectively; $n = 4-6$).

Figure 3. Voltage-dependence of Ca^{2+} inhibition

Representative traces and associated amplitude frequency histograms of outside-out patch single channel currents evoked in solutions with no-added Ca^{2+} (a), and 2 mM $[\text{Ca}^{2+}]_o$ (b) at a range of membrane potentials. For traces recorded at -80 to +40 mV, the x and y axes on the scale bars are 200 ms and 5 pA respectively. For traces recorded at +80 mV, the x and y axes on the scale bars are 2 s and 5 pA respectively. (c) Graph charting relationship between mean unitary current amplitude and membrane potential in the absence and presence of varying $[\text{Ca}^{2+}]_o$. (d) Relationship between normalized conductance, membrane potential and $[\text{Ca}^{2+}]_o$. Conductances were calculated from the data in (c) and normalized to the control conductances observed in the absence of added extracellular Ca^{2+} .

Figure 4. Ca^{2+} - and voltage-dependence of fractional Ca^{2+} current ($Pf\%$)

(a and c) Panels show representative traces of capsaicin-activated whole-cell inward current (left), and the contribution of Ca^{2+} (right, shaded area) to the total charge entering during current activation (right, solid line) as determined using patch clamp photometry. (a) The $Pf\%$ was determined from TRPV1-mediated currents evoked at a fixed membrane holding potential of -60 mV in a range of $[Ca^{2+}]_o$. (b) The relationship between $[Ca^{2+}]_o$ and $Pf\%$ values was adequately described by the Goldman-Hodgkin-Katz constant field model (solid line). (c) The $Pf\%$ was also determined from TRPV1-mediated currents activated in $2 \text{ mM } [Ca^{2+}]_o$ at a range of membrane holding potentials. (d) The relationship between holding potential and $Pf\%$ was observed to deviate from GHK predictions at depolarized potentials (solid line).

Figure 5. Effect of mutating pore-lining acidic amino acids on Ca²⁺-dependent inhibition
(a) Schematic representation of a TRPV1 in the plasma membrane (based on Shaker Kv1.2 channel architecture [43]). Putative pore-lining acidic side chains are shown in red. **(b)** Representative single channel current traces and associated frequency histograms for TRPV1-E636Q/D646N recorded in different concentrations of extracellular Ca²⁺. **(c)** Histogram showing the persistence of [Ca²⁺]_o (2–10 mM)-dependent inhibition in all mutant receptors bearing substitutions at one or more of the positions shown in Panel **(a)**. The double mutant TRPV1-E636Q/D646N is represented by QN, and the triple mutant, TRPV1-D646N/E648Q/E651Q, by NQQ. Asterisks indicate values significantly different from respective control values (0 Ca²⁺).

Figure 6. TRPV1-NQQ carries a substantially reduced Ca^{2+} current

Representative traces show inward currents activated by capsaicin (**a and c, left**) or ATP (**b, left**), and the contribution of Ca^{2+} (right, shaded area) to the total charge entering during the current activation (right, solid line) as determined using patch clamp photometry. **a and b**) In the presence of 2 mM $[Ca^{2+}]_o$, HEK293 cells co-expressing TRPV1-NQQ and the ATP-gated P2X2 receptor were stimulated with capsaicin (10 μ M), which evoked an inward current but not a measurable influx of Ca^{2+} (**a**). To control for the possibility that the lack of measurable Ca^{2+} influx was due to saturation of intracellular fura-2, we subsequently stimulated these same cells with ATP (30 μ M) to activate Ca^{2+} permeable P2X receptors, which elicited currents coupled with a measurable influx of Ca^{2+} (**b**). **c**) In the presence of

10 mM $[Ca^{2+}]_o$, stimulation of HEK293 cells expressing TRPV1-NQQ with capsaicin evoked an a measurable Ca^{2+} influx that corresponded to a Pf% of 12.3 ± 0.8 (n=3).

Figure 7. Inhibition of TRPV1-NQQ conductance could not be as easily relieved by hyperpolarization in cells expressing TRPV1-NQQ mutant

a and b) Representative traces and associated amplitude frequency histograms of outside-out patch TRPV1-WT- (**a**) and TRPV1-NQQ-mediated (**b**) currents evoked in an external solution of 10 mM $[Ca^{2+}]_o$ at a range of membrane potentials. **(c)** Graph charting relationship between mean unitary current amplitude and membrane potential. The dashed lines represent the current-voltage curves obtained in the absence of extracellular Ca^{2+} for TRPV1-WT (black) and TRPV1-NQQ (red). The solid lines represent the current-voltage curves obtained in 10 mM $[Ca^{2+}]_o$ for TRPV1-WT (black) and TRPV1-NQQ (red). Asterisks indicate TRPV1-NQQ current amplitude data that is significantly different from the obtained for the wild type at the same membrane potentials. Unitary current amplitudes could not be obtained at -120 mV in the absence of divalent cations for either wild-type or TRPV1-NQQ due to a substantial breakdown in patch integrity.

Table 1

Comparison of chord conductance data (g) obtained from single channel experiments with relative permeability (P_X/P_{Na}) data obtained from whole-cell reversal potential experiments conducted under bi-ionic conditions. N numbers are shown in brackets.

Cation	g	P_X/P_{Na}
Na⁺	62.8 ± 4.3 (25)	1
Li⁺	55.7 ± 4.6 (5)	0.84 ± 0.06 (5)
K⁺	53.1 ± 2.3 (8)	1.05 ± 0.03 (6)
Cs⁺	54.7 ± 2.4 (4)	0.94 ± 0.08 (5)
Mg²⁺	14.6 ± 1.1 (4)	5.5 ± 0.5 (5)
Ca²⁺	15.2 ± 0.7 (6)	10.2 ± 0.8 (13)