Voltage-dependent Changes of TRPV6-mediated Ca2+ Currents*

Received for publication, September 7, 2004, and in revised form, December 2, 2004
Published, JBC Papers in Press, December 6, 2004, DOI 10.1074/jbc.M410184200

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The physiological role and activation mechanism for most proteins of the transient receptor potential (TRP) family are unknown. This is also the case for the highly Ca2+-selective transient receptor potential vanilloid type 6 (TRPV6) channel. Patch clamp experiments were performed on transiently transfected human embryonic kidney (HEK) cells to address this issue. Currents were recorded under various conditions of intracellular Ca2+ buffering and monitored at the same voltage throughout. No TRPV6-mediated Ca2+ entry was detected under in vivo Ca2+ buffering conditions at a slightly negative holding potential; however, moderate depolarization resulted in current activation. Very similar results were obtained with different Ca2+ chelators, either EGTA or BAPTA dialyzing the cell. TRPV6 channel activity showed a negative correlation with the intracellular free Ca2+ concentration ([Ca2+]i), and was modulated by the membrane potential: Hyperpolarization decreases and depolarization increases TRPV6-mediated currents. Monovalent ions permeated TRPV6 channels in the absence of extracellular divalent cations. These currents were resistant to changes in the holding potential while the negative correlation to the [Ca2+]i was conserved, indicating that the voltage-dependent current changes depend on blocking and unblocking the charge carrier Ca2+ within the pore. In summary, these results suggest that the voltage dependence of TRPV6-mediated Ca2+ influx is of physiological importance since it occurs at cytosolic Ca2+ buffering and takes place within a physiologically relevant membrane potential range.

The transient receptor potential (TRP)† Drosophila melanogaster mutant was discovered by its defective visual response to prolonged illumination, which in Drosophila is a phospholipase C-dependent process (1). The gene product functions as a Ca2+-permeable channel, and Drosophila deficient in the TRP protein lack sustained Ca2+ entry (2). Since the cloning of the original TRP gene (3), several mammalian homologues have been described. They can be divided into at least three subfamilies, based on similarities in the structures of the encoded proteins: the TRPC, TRPM, and TRPV group (4–6).

Within the TRPV group, TRPV5 and TRPV6 channels show exclusive Ca2+ selectivity and are thought to be responsible for Ca2+ uptake in the kidney and intestine, respectively (7, 8). The TRPV6 protein functions as a Ca2+-sensing Ca2+ pore in HEK and RBL cells and its current amplitude is inversely correlated with the [Ca2+]i (9). However, the calculated [Ca2+]i necessary for channel activity are several magnitudes below physiological values (9). Furthermore, even high concentrations of fast exogenous Ca2+ chelators are unable to control the microdomain in the close proximity of the channel (9). Thus, the physiological activation mechanism of TRPV6 is not clear because no current was detected under in vivo Ca2+ buffering conditions (10).

In the present study the role of the membrane potential in modulating TRPV6-mediated Ca2+ currents was studied in TRPV6-transfected HEK cells. Moderate changes in the holding potential within the physiological range induced current augmentation at depolarization and current inhibition at hyperpolarization under in vivo conditions of intracellular Ca2+ buffering. This effect is caused by Ca2+ itself, which most likely binds to the TRPV6 protein in a voltage-dependent manner.

**EXPERIMENTAL PROCEDURES**

Cell Culture, Transfected cDNA, and Transfection—HEK-293 (ATCC, 1573-CRL) cells were from the American Type Culture Collection (Manassas, VA). Cell culture was done as described previously (9). Cells were transiently transfected with 4 μg of DNA in 5 ml of the PolyFect® reagents (Qiagen, Hilden, Germany). The bicistronic expression plasmid pdiCaT-Lb was constructed as described (11) and contained the entire protein-coding regions of the b-variant of human TRPV6 (formerly CaT-Lb, DDBJ/EMBL/GenBankTM, accession number CAC20417) followed by an internal ribosomal entry side and the green fluorescence protein DNA.

Site-directed Mutagenesis—Mutagenesis of single amino acids in the presumable core region were carried out using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) as reported previously for the TRPV6D542A point mutant (10). For the double mutant two complementary oligonucleotides introduced alanine residues at positions 542 and 550 (sense primer, 5′-CAT CAT CGG TGG CCC AGC CAA CTA CAA CGT GGC CCT GCC TTC C-3′ and antisense primer, 5′-AGG AGC AGC GCC AGC AGC TAT GAT ATG TGG CCA GCC AGC ATG G-3′). The mutated TRPV6 PCR products were excised from the pcDNA3 vector (Invitrogen, Karlsruhe, Germany) and subcloned into the pcAGGS-ires-GFP vector. The nucleotide sequence of the complete inserts including the Kozak sequence and the cloning sites were verified by sequencing the corresponding DNAs on both strands. The truncated construct TRPV6D542A-725 and the TRPV6D542A250 mutant have been described previously (12).

**Electrophysiological Recordings and Solutions**—Patch clamp experiments were performed in the whole cell configuration (13) using an EPC-9 amplifier (HEKA Elektronik, Lambrecht, Germany). Cells were measured 24–32 h after transfection in a modified Ringer’s solution containing (in mM): 145 NaCl, 10 CaCl2, 10 CsCl, 2.8 KCl, 2 MgCl2, 11 glucose, 10 HEPES, adjusted to pH 7.2 with NaOH. The divalent-free (DFV) solution contained (in mM): 145 NaCl, 2.8 KCl, 10 CaCl2, 11 glucose, 10 EGTA, 10 HEPES, adjusted to pH 7.2 with NaOH. The external solution for the optical recordings contained either 2 mM Ca2+ or 1 mM EGTA. Extracellular solution changes were made by pressure ejection (~5 cm H2O) from a wide-tipped pipette positioned about 10 μm from the cell. Patch pipettes pulled from borosilicate glass (Kimax®).
had resistances between 2 and 3 MΩ when filled with the standard internal solution. This solution contained (in mM): 145 Cs-glutamate, 10 HEPES, 8 NaCl, 1 MgCl₂, 2 Mg-ATP adjusted to pH 7.2 with CsOH. The free Mg²⁺ concentration was clamped by the addition of 10 mM EGTA and 3.64 mM CaCl₂ to the Cs-glutamate-based pipette solution. The nominally Mg²⁺-free solution contained (in mM): 145 Cs-glutamate, 10 HEPES, 10 EGTA, 6 Na₂-ATP, 8 NaCl adjusted to pH 7.2 with CsOH. The intracellular Mg-ATP concentration was increased to 6 mM for experiments in the absence of external divalent cations. Perforated patch recordings were performed with the standard intracellular solution supplemented with 100 μg/ml nystatin (14). The tips of the patch pipettes were filled with nystatin-free internal solution by capillary force. When challenged with step depolarizations, perforation of the membrane patch was indicated by characteristic changes of the capacitance transient and by a continuous decline of the input resistance. To refine the membrane patch was indicated by characteristic changes of the capacitance transient and by a continuous decline of the input resistance. To

The intracellular Mg-ATP concentration was increased to 6 mM for such negative potentials (Fig. 1 through the activation of voltage-dependent channels, which caused by a basal Cl⁻ conductance present in these cells (15). The inward current at potentials around 30 mV may arise through the activation of voltage-dependent channels, which are expressed in this cell line (16) and are not deactivated at such negative potentials (Fig. 1B, top).

At a holding potential of −10 mV the initial inward current measured by the first voltage ramp was larger than at more hyperpolarized membrane potentials (−2.3 ± 0.8 pA/pF, n = 10 at −80 mV and −8.2 ± 2.4 pA/pF, n = 7 at −10 mV). The current increased in size, peaked after 10–20 s of whole cell recording and subsequently decayed until a steady-state level was reached (Fig. 1A, middle). It was inwardly rectifying and reversed at positive potentials indicating a high C₅⁺ selectivity (Fig. 1B, middle). No such current was recorded in the absence of external Ca²⁺ from TRPV6-transfected HEK cells (data not shown, n = 5). Likewise, this current was not detected in either mock or non-transfected HEK cells (data not shown, n = 5 each).

Depolarization of the membrane resulted in a further increase of the TRPV6-mediated Ca²⁺ entry (−26.4 ± 3.3 pA/pF, n = 7 at −10 mV and −47.8 ± 7.8 pA/pF, n = 12 at 50 mV). The time course and voltage dependence are shown at the bottom of Fig. 1. Taken together, these results indicate that the size of TRPV6-mediated Ca²⁺ currents are strongly dependent on the holding potential, as suggested previously (10, 11, 17, 18).

Current Changes during Voltage Steps of TRPV6-expressing HEK Cells with High Intracellular Ca²⁺ Buffering—To test whether the voltage-dependent modulation of TRPV6 channel activity depends on the preceding state of channel activation or inactivation, the holding potential was changed during the recording from the same cell. With 10 mM EGTA in the pipette solution and a holding potential of −10 mV the Ca²⁺ current activated rapidly. After the peak amplitude was reached at about 30 s, the current inactivated until current densities were stable at about 200 and 300 s. After this time, the cell is expected to be almost completely dialedyzed with the EGTA containing intracellular solution (19). Next, the cell membrane was clamped at 50 mV between voltage ramps. As a consequence the current amplitude slowly increased with an exponential time course (τ = 58 s). After stepping back to −10 mV, the Ca²⁺ entry rapidly decayed in a complex manner, before current densities were reconstituted to values similar to the ones recorded before depolarization (Fig. 2A). This voltage-de-
containing 10 mM EGTA. The membrane potential was changed from −10 mV to 50 mV as indicated. The correlating changes in current density at −80 mV and 80 mV are plotted versus time. B, I-V relationships were recorded with the ramp protocol shown at the top. At the time indicated in A current traces were measured. Note the different scaling of the current density in 2. A typical example is shown for five similar recordings.

Fig. 3. Tail and steady-state currents obtained from voltage steps. Tail currents were measured during the first milliseconds of the voltage steps and steady-state currents were determined at the end of the voltage pulses. The voltage protocol and the corresponding current traces from representative cells are shown in the inset. The membrane potential was either −10 mV (A) or 50 mV (B). Currents were measured after their amplitude was stable, which was detected by the repetitive application of voltage ramps similar to the recording shown in Fig. 2. TRPV6-expressing HEK cells were dialyzed with the Cs-glutamate-based internal solution containing 10 mM EGTA. The modified Ringer’s solution with 10 mM Ca2+ was used extracellularly. Current densities were normalized to their maximal current at −110 mV and plotted versus voltage (n = 13).

dependent modulation of Ca2+ entry was reversible because it was recorded several times from the same cell (data not shown, n = 20). Intracellular diffusible factors do not seem to be responsible for this effect because there was no correlation with the duration of whole cell perfusion. It was possible to elicit these changes in current densities with respect to the holding potential at physiological potentials. Depolarization increased the TRPV6-mediated Ca2+ currents and hyperpolarization had the opposite effect (data not shown, holding potentials of −110 to 90 mV tested).

High resolution Ca2+ currents evoked by voltage ramps after maximum activation at −10 and 50 mV holding potential are shown in Fig. 2B. On the basis of the similar I-V characteristics it is most likely that only TRPV6-mediated Ca2+ influx was measured without major contamination of other currents. This voltage protocol was used in the following experiments to study the effect of the holding potential on the TRPV6 channel in more detail.

Other members of the TRP family, namely TRPV1 and TRPM8 have recently been shown to behave in a voltage-dependent manner. Both cation channels can be activated at different temperatures and thereby current activation at depolarized potentials precedes that at more negative potentials (20). However, such a behavior was not detected for the strongly inward rectifying TRPV6-mediated Ca2+ current when activated with EGTA dialysis or membrane depolarization (Fig. 2A). Likewise, no obvious changes in the voltage dependence of TRPV6-mediated Ca2+ influx was recorded at the two holding potentials with a tail current protocol (Fig. 3).

Current Changes during Voltage Steps of TRPV6-expressing HEK Cells at Low Intracellular Ca2+ Buffering—It has been previously shown, that TRPV6 channel activity correlates with the intracellular Ca2+ chelator concentration (9). However, the calculated global [Ca2+]i, at which TRPV6 channels start to activate is extremely low and several magnitudes below physiological values (9). Therefore, it is crucial to find out whether TRPV6 channels open because of changes in membrane potential under more physiological conditions.

At moderate cytoplasmic Ca2+ buffering obtained by dialyzing cells with low concentrations of EGTA (0.1 mM), no current developed at a holding potential of −10 mV (−1.7 ± 0.3 pA/pF, n = 12; Fig. 4A). In contrast, large Ca2+ currents were recorded at higher intracellular EGTA concentrations (Figs. 1 and 2) indicating that TRPV6 channels are strongly dependent on [Ca2+]i, and are significantly augmented by decreasing [Ca2+]i. Switching the holding potential from −10 to 50 mV led to a gradual current increase until steady state was reached (−8.9 ± 0.8 pA/pF, n = 3; Fig. 4A). This current is likely to be TRPV6-mediated since it was strongly inward rectifying and reversed at positive potentials. Repolarization from 50 to −10 mV resulted in current decay and a very similar level was reached compared with the one before depolarization. Therefore, it is indeed possible to demask TRPV6-mediated Ca2+ entry under quite physiological conditions of intracellular Ca2+ buffering.
buffering and membrane potentials. In non-transfected HEK cells no such current and voltage-dependent behavior was seen (Fig. 4A).

The intracellular Ca\(^{2+}\) dependence of TRPV6 channels with respect to its voltage-dependent behavior was further investigated with an internal solution containing 100 mM free Ca\(^{2+}\) using the appropriate concentration of EGTA (10 mM) and CaCl\(_2\) (3.64 mM; Fig. 4B). Again, no current activation was detected like in the experiments using 0.1 mM EGTA. In contrast to the experiments with 0.1 mM EGTA, the very same depolarization of the plasma membrane was now unable to activate TRPV6-mediated Ca\(^{2+}\) entry (−0.7 ± 0.3 pA/pF; n = 3; Fig. 4B). Thus, the TRPV6-mediated Ca\(^{2+}\) current density depends on both the intracellular EGTA concentration and the membrane potential (Fig. 4A).

Perforated patch clamp recordings do not alter the endogenous Ca\(^{2+}\) buffering because there is no washout of cytosolic components (14). Therefore, nystatin patches were used to find out whether changes in the membrane potential can modulate TRPV6-mediated Ca\(^{2+}\) entry in a situation with physiological Ca\(^{2+}\) buffering. This was indeed the case as shown in Fig. 4, C–E. The voltage step from −10 to 50 mV induced the slow activation of an inward current with the typical voltage dependence of the TRPV6 protein (−1.9 ± 0.5 pA/pF; n = 5; Fig. 4C). No such current was recorded either before or after depolarization at the holding potential of −10 mV (Fig. 4, C–E). Likewise, no inward rectifying Ca\(^{2+}\) current was measured in non-transfected HEK cells if voltage ramps were applied from either −10 or 50 mV (−0.6 ± 0.1 pA/pF; n = 4; Fig. 4C). In summary, these results favor the idea that TRPV6-mediated Ca\(^{2+}\) currents are regulated in a voltage-dependent manner under in vivo Ca\(^{2+}\) buffering conditions.

Voltage-dependent Modulation of TRPV6-mediated Ca\(^{2+}\) Currents Is Dependent on Intracellular Ca\(^{2+}\). The preceding data demonstrate that the amplitude of TRPV6-mediated Ca\(^{2+}\) currents is reduced as the holding potential between ramps becomes negative and is increased at positive voltages. It might be possible that cations bind closely to or even within the pore and thereby plug the channel. During membrane depolarization the blocking particle is electrostatically removed, which favors Ca\(^{2+}\) entry. However, voltage-dependent current changes were relatively slow in comparison to the rapid changes in the holding potential. The following experiments should clarify the underlying mechanism for this voltage-dependent modulation of TRPV6 channel gating.

Negative membrane potentials provide a favorable driving force for Ca\(^{2+}\) entry and it is conceivable that the enhanced Ca\(^{2+}\) influx could inactivate TRPV6 channels in a Ca\(^{2+}\)-dependent manner. This Ca\(^{2+}\)-dependent inactivation arises from the build-up of a microdomain of Ca\(^{2+}\) in the vicinity of each channel (9). This local Ca\(^{2+}\) gradient might be controlled if high concentration of a fast Ca\(^{2+}\) chelator (60 mM BAPTA) were dialyzed into the cell (Fig. 6). TRPV6 channel activation was monitored by sporadic application of short voltage ramps (25 ms duration, every 10 s). Under these conditions Ca\(^{2+}\) currents were significantly larger (−169 ± 21 pA/pF; n = 10 versus −38 ± 11 pA/pF; n = 6 after 200 s whole-cell recording) and activated faster (τ = 25 ± 2 s, n = 11 versus τ = 36 ± 5 s, n = 6) than with the standard voltage protocol (50 ms duration, every 2 s). Steady-state levels were reached within 100 s and no rundown was detected (Fig. 6). After about 200 s of whole cell perfusion the intracellular BAPTA concentration is thought to be roughly the same as in the pipette solution (19). Now the repetitive voltage ramps were no longer applied to minimize Ca\(^{2+}\) entry to allow an efficient Ca\(^{2+}\) buffering close to the TRPV6 pore. After a pause of 1 min, Ca\(^{2+}\) current amplitude did not increase, suggesting that BAPTA was able to achieve an equilibrium within the presumed Ca\(^{2+}\) microdomain responsible for inactivation during 10 s or
less (Fig. 6B). Thus, Ca\(^{2+}\) currents seemed to be maximally activated, but switching the holding potential from −10 to 50 mV resulted in an augmentation of Ca\(^{2+}\) influx without changes in the voltage dependence (Fig. 6B). These data suggest that the presumable Ca\(^{2+}\) binding site within the TRPV6 protein responsible for the voltage-dependent modulation of the Ca\(^{2+}\) currents may not be accessible from the cytosolic side. Another possibility is that it binds Ca\(^{2+}\) with such a high affinity that the chelator cannot interfere.

**Voltage-dependent Modulation of TRPV6-mediated Ca\(^{2+}\) Currents with Varying Intracellular Mg\(^{2+}\)—**Intracellular Mg\(^{2+}\) can block K\(^+\) channels in a voltage-dependent manner thereby inducing an inward rectification (21). In analogy, Mg\(^{2+}\) ions might plug TRPV6 channels from the inside at hyperpolarized potentials. At positive potentials this block then might be removed leading to enhanced Ca\(^{2+}\) entry. This idea was tested by examining the effects of Mg\(^{2+}\)-free conditions on the voltage-dependent modulation of Ca\(^{2+}\) currents (Fig. 7). TRPV6-expressing HEK cells were dialyzed with a nominally Mg\(^{2+}\)-free internal solution. Switching the holding potential from −10 to 50 mV after 300 s of whole cell perfusion resulted in a 5.4-fold current increase (−4.4 ± 1.7 pA/pF to −23.9 ± 7.1 pA/pF, n = 4; after initial conductance correction). Thus, dramatically reducing the intracellular Mg\(^{2+}\) concentration did not abolish the inward rectification and voltage-dependent modulation of Ca\(^{2+}\) currents in TRPV6-expressing HEK cells. This was also the case when neither Mg\(^{2+}\) nor ATP was added to the internal solution excluding a protein kinase-mediated effect (data not shown, n = 4).

**Voltage-dependent Modulation of TRPV6-mediated Ba\(^{2+}\) Currents—**As shown above the voltage-dependent behavior of TRPV6 channels was not abolished when either the intracellular Mg\(^{2+}\) or Ca\(^{2+}\) concentration was decreased dramatically. Therefore, Ba\(^{2+}\) was used next as the charge carrier instead of Ca\(^{2+}\) because Ba\(^{2+}\) permeates Ca\(^{2+}\) channels but substitutes poorly at Ca\(^{2+}\)-binding proteins. Replacing Ca\(^{2+}\) by Ba\(^{2+}\) in the bath solution resulted in similar inward currents compared with the experiments with 10 mM intracellular EGTA (peak current densities: −27.9 ± 4.7 pA/pF, n = 7 for \(I_{\text{Ba}^{2+}}\); and −23.8 ± 4.8 pA/pF, n = 6 for \(I_{\text{Ca}^{2+}}\)). Likewise, the effect of holding potential was conserved switching from Ca\(^{2+}\) to Ba\(^{2+}\) currents (Fig. 8). Thus, Ba\(^{2+}\) is able to substitute for the physiological charge carrier without abolishing the voltage dependence on current amplitude.

**Voltage-dependent Modulation of TRPV6-mediated Monovalent Currents—**Given the presumable importance of the charge carrier it was decided to record monovalent currents in the absence of extracellular divalent cations (Fig. 9). TRPV6-expressing HEK cells were dialyzed with 10 mM EGTA to activate prominent Ca\(^{2+}\) influx. The holding potential was changed after 300 s of whole cell recording for 120 s and induced the typical augmentation of Ca\(^{2+}\) entry as previously shown. Under DVF conditions the inward current was initially depressed, as one would expect for anomalous mole fraction behavior. Subsequently, large monovalent currents developed with an exponential time course, reached their peak amplitudes, which stayed stable with time. Now the holding potential was changed again, but without any effect on current density. After the application of the DVF solution was stopped, the inward current decayed rapidly. The very same depolarization of the membrane potential was now able to increase the TRPV6-mediated Ca\(^{2+}\) current. I-V relationships allowed the identification of the typical TRPV6-mediated currents in the presence or absence of external divalent cations (Fig. 9B). Ca\(^{2+}\) and monovalent currents were elicited by standard voltage ramps from a holding potential of −10 mV immediately before depolarization to 50 mV, at the end of the 120-s interval at 50 mV and after repolarization to −10 mV. In all cases the inward current reversed at slightly positive potentials and showed the characteristic negative slope below −80 mV, which is due to time-dependent removal of an intracellular Mg\(^{2+}\) block (9, 22).

Strikingly, no increase in monovalent current amplitude was detected due to the changes in membrane potential (Fig. 9). These data suggest that the charge carrier is crucial in generating the voltage-dependent conductance changes of the TRPV6 channel. In addition, channel activity is still dependent on [Ca\(^{2+}\)], even if monovalent ions instead of Ca\(^{2+}\) permeate the TRPV6 pore (Fig. 5B).

**Voltage-dependent Modulation of TRPV6 Channels Is Independent of Calmodulin Binding at the C Terminus and Ca\(^{2+}\)-
By voltage ramps from changed after 280 s from their voltage dependence. No voltage protocol was applied for 1 min were scaled to their maximal amplitude to allow a better comparison of curves are shown at the four time points as indicated. Current traces with 60 mM BAPTA (Hz).

FIG. 6. TRPV6-mediated Ca\textsuperscript{2+} currents at high intracellular concentrations of the fast Ca\textsuperscript{2+} chelator BAPTA elicited by different voltage protocols and holding potentials. A, time course of currents in TRPV6 expressing HEK cells perfused with an intracellular solution counting 60 mM BAPTA. Voltage ramps spanning from −110 mV to 90 mV in 50 or 25 ms were applied every 2 or 10 s. Mean data with double-sided S.E. are shown (n = 6 for the voltage protocol with ramps or 50 s duration at 0.5 Hz and n = 10 for the 25 ms ramps at 0.1 Hz). B, typical current trace of a TRPV6 expressing HEK cell dialyzed with 60 mM BAPTA (n = 4). Inward currents were monitored every 10 s by voltage ramps from −110 to 90 mV within 25 ms. Representative I-V curves are shown at the four time points as indicated. Current traces were scaled to their maximal amplitude to allow a better comparison of their voltage dependence. No voltage protocol was applied for 1 min from 190 to 250 s of whole cell recording. The membrane potential was changed after 280 s from −10 to 50 mV as illustrated.

FIG. 7. Voltage-dependent modulation of TRPV6 channels in the absence of intracellular Mg\textsuperscript{2+}. TRPV6 transfected HEK cells were dialyzed with an Mg\textsuperscript{2+}-free solution containing 10 mM EGTA. Standard voltage ramps were applied from a holding potential of either −10 or 50 mV as indicated. Averaged inward currents are plotted with double-sided S.E. versus the duration of whole cell recording (n = 4).

Binding to the Aspartate Residue at Position 542 within the Presumable Pore Region—The results shown so far indicate a divalent cation-dependent mechanism responsible for TRPV6 channel activity. Previously it was shown that Ca\textsuperscript{2+}-dependent CaM binding at the C terminus of TRPV6 facilitates channel inactivation, which can be counteracted by PKC-mediated phosphorylation of a threonine residue at position 702 within this CaM binding site (12). Therefore, the following experiments were designed to test whether CaM and PKC contribute to the voltage-dependent modulation of TRPV6 channels.

The CaM binding site covering amino acid residues 694–716 of TRPV6 is absent in the truncated TRPV6\textsubscript{Δ693–725} variant, which lacks the C-terminal 33 amino acid residues including the CaM binding site (12). However, the typical Ca\textsuperscript{2+} current increase was elicited by changing the holding potential from −10 to 50 mV (Fig. 10A). Similar results were obtained at lower intracellular Ca\textsuperscript{2+} buffering (0.1 mM EGTA instead of 10 mM, data not shown, n = 3). Replacing the threonine residue at position 702 by an alanine residue (TRPV6\textsubscript{T702A}), does not affect CaM binding but the TRPV6 protein can no longer be phosphorylated at this site (12). However, the effect of the membrane potential on Ca\textsuperscript{2+} current amplitude was also present when the TRPV6\textsubscript{T702A} variant was expressed (data not shown, n = 6). Similar recordings were performed on wild-type TRPV6 expressing HEK cells incubated with the broad spectrum kinase blocker staurosporine (2 μM for at least 30 min). In addition, ATP was omitted from the EGTA (10 mM) containing pipette solution to further reduce kinase activity. Under these conditions PKC activity is expected to be extremely low; however, Ca\textsuperscript{2+} current changes in response to the membrane potential remained (data not shown, n = 5). Thus, neither CaM binding at position 693–725 nor PKC-mediated phosphorylation at Thr\textsuperscript{702} was responsible for the voltage-dependent conductance changes.

Previously it has been shown that the aspartate residue at position 541 of the mouse TRPV6 protein determines Ca\textsuperscript{2+} permeation and Mg\textsuperscript{2+} block (23). The mutation of the corresponding aspartate residue to alanine in the human TRPV6 yields proteins inserted in the plasma membrane, which do not function as ion conducting channels (10). Also switching the holding potential from −10 mV to 50 mV did not allow the detection of Ca\textsuperscript{2+} currents in HEK cells expressing the TRPV6\textsubscript{T542A} protein (Fig. 10B). Likewise, no differences to non-transfected cells were detected from cells expressing the double mutant where the aspartate residue at position 542 and the glutamate residue at position 550 were mutated into alanine residues (data not shown, n = 4).

DISCUSSION

The present study shows that the dose response curve for TRPV6-mediated Ca\textsuperscript{2+} currents in dependence of the [Ca\textsuperscript{2+}], (9) is shifted by the membrane potential: Depolarization induced an augmentation of Ca\textsuperscript{2+} entry whereas hyperpolarization reduced current densities. No prominent Ca\textsuperscript{2+} current was detected if TRPV6-expressing HEK cells were clamped at −10
FIG. 9. TRPV6-mediated Ca\(^{2+}\) and monovalent Ca\(^{2+}\) currents at various holding potentials. A, time course of currents from a representative TRPV6-expressing HEK cell dialyzed with an intracellular solution containing 10 mM EGTA (n = 6). The membrane potential was clamped at −10 or 50 mV as shown on the top. The normal external solution was substituted versus DVF saline, as indicated with the bar. B, I-V relations were recorded before, during, and after depolarization at the time points highlighted in A.

![Image](103x343 to 261x527)

FIG. 10. Voltage-dependent modulation of Ca\(^{2+}\) currents for a calmodulin binding site deletion construct, a disabled phosphorilation site mutant and a point mutant in the presumable pore region of the TRPV6 protein. HEK cells expressed two different mutant channels: The deletion construct of TRPV6 lacked amino acids 693–725 (A) and the point mutant contained an alanine at position 542 instead of an aspartate in the wild type (B). The intracellular solution contained 10 mM EGTA. Mean data are shown (A, n = 7; B, n = 14).

mV between voltage ramps and whole cell recordings were performed with 0.1 mM intracellular EGTA. Increasing either the membrane potential or the chelator concentration resulted in the activation of TRPV6 channels with their characteristic I-V signature. Likewise, no TRPV6-mediated current was detected with 10 mM EGTA during hyperpolarization whereas the typical TRPV6 current developed if the holding potential became positive. Thus, the TRPV6 protein functions as a Ca\(^{2+}\) channel and its current amplitude is strongly dependent on both [Ca\(^{2+}\)\(^+\)] and membrane potential.

The voltage-dependent modulation of TRPV6 channels is an unexpected finding since the driving force for Ca\(^{2+}\) was only changed between pulses and currents were measured at the same potential throughout by applying voltage ramps repetitively. One explanation for these data is that due to membrane depolarization a blocking particle is removed from the pore. Unlike inward rectifying K\(^+\) channels and NMDA receptors, it does not seem to involve voltage-dependent block by intracellular Mg\(^{2+}\). This result is of particular interest since it has been previously shown that Mg\(^{2+}\) can block TRPV6 channels in a voltage-dependent manner (9, 22). The role of Ca\(^{2+}\) ions, which may plug the TRPV6 channel from the cytosolic side was examined in experiments with 60 mM BAPTA in the pipette solution. As previously reported, current densities did not reach steady state but continuously increased in comparison to current amplitudes recorded with 30 mM BAPTA (9). Changing the holding potential affected the TRPV6-mediated Ca\(^{2+}\) current suggesting that intracellular Ca\(^{2+}\) is not important for this effect. Another possibility is that even these high concentrations of a fast chelator are unable to access the putative Ca\(^{2+}\) binding site at the pore and thereby relieve channels from partial Ca\(^{2+}\)-dependent inactivation. Since the changes in current density could be repeatedly evoked during prolonged whole cell recordings, it is unlikely that a small diffusible factor could be involved. Such a molecule would be effectively washed out of the cell into the recording pipette.

The mechanism by which TRPV6 channels operate in a voltage-dependent manner involves the charge carrier. Like Ca\(^{2+}\) currents, Ba\(^{2+}\) currents were augmented during depolarization and reduced at hyperpolarization. However, in the absence of divalent external cations monovalent ions permeate the TRPV6 pore. These currents, mainly carried by Na\(^+\) ions, were independent to changes in the holding potential. Therefore, one might imagine a scenario in which permeating Ca\(^{2+}\) ions block the pore under physiological conditions. Depolarization of the cell membrane could result in electrostatic repulsion of these Ca\(^{2+}\) ions, thus facilitating Ca\(^{2+}\) flux and \textit{vice versa} at hyperpolarization. Stepping to 50 mV, the Ca\(^{2+}\) current usually developed slowly and decayed rapidly when returning back to −10 mV. The different kinetics probably reflect the regulation of TRPV6 by Ca\(^{2+}\) that may dissolve slowly but rebind rapidly. With this idea in mind, the slower kinetics of Ba\(^{2+}\) current activation and inactivation could be explained as resulting from the different binding affinities of Ba\(^{2+}\) and Ca\(^{2+}\) to the pore. Both, TRPV6-mediated Ca\(^{2+}\) and monovalent currents show a negative correlation of amplitude and [Ca\(^{2+}\)]\(^+\). However, only Ca\(^{2+}\) influx, but not monovalent currents can be modulated by the membrane potential. Thus, both processes are regulated independently.

Voltage-operated Ca\(^{2+}\) channels compromise four glutamate residues in the putative pore region, which are likely to be responsible for the high Ca\(^{2+}\) selectivity (24, 25). Electrostatic interaction between these glutamate residues and Ca\(^{2+}\) are a critical determinant of high affinity Ca\(^{2+}\) binding and permeation properties, suggesting that these negative residues could behave as surrogate water molecules to facilitate the passage of dehydrated Ca\(^{2+}\) through the hydrophobic plasma membrane.
(26). However, the only negatively charged amino acid residues in the putative pore-forming region of TRPV6 are a glutamate residue at position 535 and two aspartate residues at positions 542 and 550. All three amino acids are conserved in the TRPV5 protein from rabbit and especially the aspartate residue at position 542 has been shown to decisively affect Ca\(^{2+}\) permeation (27, 28). Substituting aspartate at position 542 versus an alanine in human TRPV6 abolished channel activity (10), which was also the case if this pore mutant was C-terminally EGFP-tagged.\(^2\) However, the very same mutant of mouse TRPV6 conducted monovalent currents under physiological conditions with slightly more outward rectification than in wild-type TRPV6-transfected HEK cells, when intracellular Mg\(^{2+}\) was removed (23). These functional differences may be due to the 10% different amino acid residues between human and mouse TRPV6. Interestingly, the putative pore region of the mouse clone contained an additional aspartate residue at position 547, which is not conserved in human.

The voltage-dependent modulation of TRPV6-mediated Ca\(^{2+}\) currents takes place at physiological [Ca\(^{2+}\)]\(_i\). Changes in current amplitude were recorded at membrane potentials normally encountered under \textit{in vivo} conditions by cells expressing TRPV6. For instance, pancreatic acinar cells usually have a resting potential of about −40 mV (29–31) and muscarinic stimulation results in either depolarization or hyperpolarization, which seems to be species-dependent (29–31). These changes in the membrane potential are likely to influence TRPV6-mediated Ca\(^{2+}\) entry in a complex fashion since TRPV6 channels are activated at potentials where the driving force for Ca\(^{2+}\) is small. Furthermore the incoming Ca\(^{2+}\) is blocking TRPV6 channel activity and thereby protecting the cell from toxic Ca\(^{2+}\) overload. It will be interesting to investigate whether the voltage-dependent effect on TRPV6-mediated Ca\(^{2+}\) influx occurs in primary cell culture within the range of physiological membrane potentials, thus modulating the [Ca\(^{2+}\)]\(_i\), and thereby cellular function such as secretion.

Acknowledgments—I thank Veit Flockerzi for support, encouragement, and comments on the manuscript. I would like to thank Elisabeth Glowatzki for critical reading of the manuscript and Heidi Löhr for technical assistance.

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