Hydrolysis of Phosphatidylinositol 4,5-Bisphosphate Mediates Calcium-induced Inactivation of TRPV6 Channels

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TRPV6 is a member of the transient receptor potential superfamily of ion channels that facilitates Ca\(^{2+}\) absorption in the intestines. These channels display high selectivity for Ca\(^{2+}\), but in the absence of divalent cations they also conduct monovalent ions. TRPV6 channels have been shown to be inactivated by increased cytoplasmic Ca\(^{2+}\) concentrations. Here we studied the mechanism of this Ca\(^{2+}\)-induced inactivation. Monovalent currents through TRPV6 substantially decreased after a 40-s application of Ca\(^{2+}\), but not Ba\(^{2+}\). We also show that Ca\(^{2+}\), but not Ba\(^{2+}\), influx via TRPV6 induces depletion of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P\(_2\)) or PIP\(_2\) and the formation of inositol 1,4,5-trisphosphate. Dialysis of DiC\(_8\) PI(4,5)P\(_2\) through the patch pipette inhibited Ca\(^{2+}\)-dependent inactivation of TRPV6 currents in whole-cell patch clamp experiments. PI(4,5)P\(_2\) also activated TRPV6 currents in excised patches. PI(4)P, the precursor of PI(4,5)P\(_2\), neither activated TRPV6 in excised patches nor had any effect on Ca\(^{2+}\)-induced inactivation in whole-cell experiments. Conversion of PI(4,5)P\(_2\) to PI(4)P by a rapamycin-inducible PI(4,5)P\(_2\) 5-phosphatase inhibited TRPV6 currents in whole-cell experiments. Inhibiting phosphatidylinositol 4 kinases with wortmannin decreased TRPV6 currents and Ca\(^{2+}\) entry into TRPV6-expressing cells. We propose that Ca\(^{2+}\) influx through TRPV6 activates phospholipase C and the resulting depletion of PI(4,5)P\(_2\) contributes to the inactivation of TRPV6.

Calvin signaling orchestrates a myriad of physiological functions such as muscle contraction, neurotransmitter release, bone formation, and fertilization. Ca\(^{2+}\) entry through plasma membrane ion channels regulates numerous physiological and pathophysiological processes. The essential role of transient receptor potential (TRP)\(^2\) channel proteins in the regulation of cellular Ca\(^{2+}\) signaling has begun to be appreciated in the recent past (1–3). The mammalian TRP superfamily comprises six main subfamilies named the TRPC (canonical), TRPM (melastatin), TRPV (vanilloid), TRPP (polycystin), TRPML (mucolipin), and TRPA (ankyrin) groups. Among all TRP channels, TRPV5 and TRPV6, the members of the vanilloid subfamily, are the only ones that exhibit high Ca\(^{2+}\) selectivity (4).

TRPV6 is expressed in Ca\(^{2+}\)-transporting epithelial cells, and it plays an important role in the active Ca\(^{2+}\) absorption by the intestines (5, 6). TRPV6 channels have been reported to be expressed in a variety of other tissues such as kidney, prostate, stomach, brain, and lung (7) and have also been shown to be expressed aberrantly in human malignancies (8). Until now no electrophysiological study has been performed characterizing these channels in native cells that express them endogenously. The physiological importance of these channels is understood from studies with genetically modified mice. Mice lacking TRPV6 have been shown to exhibit reduced intestinal Ca\(^{2+}\) reabsorption, increased urinary Ca\(^{2+}\) excretion, decreased bone density, reduced fertility, and skin abnormalities (9).

Electrophysiological characterization of TRPV6 channels in heterologous expression systems reveals that they exhibit strong inward rectification and reverse at positive potentials (10). They exhibit high Ca\(^{2+}\) selectivity and conduct monovalent cations in the absence of divalent cations. These monovalent currents through TRPV6 channels are much larger than those carried by Ca\(^{2+}\) at physiological Ca\(^{2+}\) concentrations (11, 12). Ca\(^{2+}\) that enters through TRPV6 or an increase in intracellular Ca\(^{2+}\) has been reported to cause inactivation of these channels (12–14). This also inactivates monovalent currents upon subsequent removal of extracellular Ca\(^{2+}\) (12). TRPV6 is also permeable to Ba\(^{2+}\), but Ba\(^{2+}\) influx induces less inactivation than Ca\(^{2+}\) or no inactivation at all depending on the conditions (12, 14). Recovery from Ca\(^{2+}\)-induced inactivation is quite slow (12), and it was shown for the closely related TRPV5 that this recovery lags significantly behind restoration of cytoplasmic Ca\(^{2+}\) levels (15). TRPV6 channels were proposed to function as Ca\(^{2+}\) sensors, i.e. at low cytoplasmic [Ca\(^{2+}\)] they open and let more Ca\(^{2+}\) in, and at high [Ca\(^{2+}\)] they close and reduce further Ca\(^{2+}\) entry (10).
Recent evidence indicates that a growing number of mammalian TRP channels are functionally regulated by PI(4,5)P₂ (16–18). Among the TRP proteins, seven are reported to be modulated by PI(4,5)P₂. The Drosophila TRPL (19) was reported to be inhibited by PI(4,5)P₂, whereas for TRPV1 both inhibition and activation were demonstrated (20–22). TRPV5 (23, 24), TRPM4 (25, 26), TRPM5 (27), TRPM7 (28, 29), and TRPM8 (23, 30) were reported to be activated by PI(4,5)P₂.

In this study we examined the role of phosphoinositides in the Ca²⁺-induced inactivation of TRPV6. We demonstrate that the activity of TRPV6 depends on PI(4,5)P₂, using different approaches including direct activation by PI(4,5)P₂ in excised patches, dialyzing DiC₈ PI(4,5)P₂ via the patch pipette in whole-cell recordings, and dephosphorylating plasma membrane PI(4,5)P₂ using a rapamycin-inducible PI(4,5)P₂-5-phosphatase. We also show that Ca²⁺ flowing through TRPV6 activates phospholipase C (PLC), which leads to the depletion of PI(4,5)P₂. Taken together, we provide evidence for a model that envisages the activation of PLC by Ca²⁺, which results in the hydrolysis of PI(4,5)P₂, causing inactivation of TRPV6 channels.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—**HEK293 cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. The human TRPV6 tagged with the Myc epitope on the N terminus, subcloned into the expression vector pCMV-Tag3A (Stratagene), was used for the experiments (31), and cells were transfected using the Effectene reagent. For the intracellular Ca²⁺ imaging and electrophysiology experiments, transfection was confirmed by measuring the fluorescence of co-transfected GFP. For experiments with rapamycin, the cells were transfected with the Myc-tagged TRPV6, the plasma membrane-targeted, CFP-tagged FRB, and the RFP-tagged FKBP12 linked to the phosphatase domain of PI(4,5)P₂-5-phosphatase (32). For control experiments, RFP-FKBP12-phosphatase domain was replaced with the RFP-tagged FKBP12 without the 5-phosphatase domain.

**Mammalian Electrophysiology—**Whole-cell patch clamp measurements were performed using a continuous holding protocol at −60 mV. Recordings were performed 36–72 h post-transfection in HEK293 cells using a bath solution containing, in mM, 137 NaCl, 5 KCl, 10 glucose, 10 HEPES, pH adjusted to 7.4 (designated as nominally divalent free, NDF). The same solution was used for the fluorescence measurements and Ca²⁺ imaging. Borosilicate glass pipettes (World Precision Instruments) of 0.8–1.7-megaohm resistance. After establishing gigaohm resistance seals on devitellinized surfaces of Xenopus oocytes, inside-out configuration was established, and currents were measured using an Axopatch 200B amplifier (Axon Instruments). The pipette solution contained, in mM, 96 KCl, 5 EGTA, 10 HEPES, pH 7.4. For the measurements shown in Fig. 3, the perfusion solution contained, in mM, 96 KCl, 5 EGTA, 10 HEPES, pH adjusted to 7.4. For the measurements shown in Fig. 7, the perfusion solution contained, in mM, 93 potassium gluconate, 5 HEDTA, 5 HEPES, pH 7.4. The free Ca²⁺ and Mg²⁺ concentrations were calculated using the MaxChelator program. To result in 10 μM free calcium, 3.85 mM Ca²⁺ was added (calcium gluconate) and to result in 43 μM free Mg²⁺, we added 2.35 mM Mg²⁺ (magnesium gluconate). For these measurements the bath was connected with the ground electrode through an agar bridge. Data were analyzed with pCLAMP 9.0 software (Axon Instruments) and plotted using Microcal Origin.

**FRET Measurements—**HEK293 cells were co-transfected with the CFP- and YFP-tagged PH domains of PLCδ1 and TRPV6. Measurements were performed using a Photon Technology International (PTI) (Birmingham, NJ) photomultiplier-based system mounted on an Olympus IX71 microscope, equipped with a DeltaRAM excitation light source. For the FRET measurements, excitation wavelength was 425 nm and emission was detected parallel at 480 and 535 nm using two interference filters and a dichroic mirror to separate the two emission wavelengths. Data were collected using the Felix software (PTI), and the ratio of traces obtained at the two different wavelengths correlating with FRET were plotted (33). Measurements were performed at room temperature (20–25°C).

**Inositol Phosphate Turnover—**Measurements were performed similarly to that described in Ref. 34. HEK293 cells were transfected with either TRPV6-Myc and GFP or with GFP alone (controls) and incubated with 20 μCi of [³²P]myo-inositol overnight in growth medium. Before the experiments the cells were kept in NDF for 20 min and for an additional 10 min in NDF containing 10 mM LiCl. Then the cells were treated with NDF containing no Ca²⁺ or 2 mM Ca²⁺ or 2 mM Ba²⁺ for 25–30 min in the continued presence of LiCl. The cells were scraped, treated with 4% perchloric acid, and centrifuged at 12000 rpm for 2–4 min. The supernatants (1.2 ml) were transferred into glass tubes containing 180 μl of 10 mM EDTA, pH 7.0, and to each tube 1.3 ml of a freshly prepared mixture of trioctylamine/Freon was added, vortexed, and centrifuged at 12000 rpm for 4 min. The top aqueous layer (~1.2 ml) was transferred into plastic vials and 3.6 ml of sodium bicarbonate was added. This solution was then added to Dowex columns filled with AG1 × 8 resin (formate form). The columns were washed four times each with 5 ml of distilled water. Fractions 1–4 (5 ml each) 0.4 M ammonium formate/0.1 M formic acid, pH 4.75 (IP₂ fraction); fractions 5–8 (5 ml each) 0.7 M ammonium formate/0.1 M for
**PIP<sub>2</sub> Regulation of TRPV6 Channels**

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**FIGURE 1. Effects of Ca<sup>2+</sup> and Ba<sup>2+</sup> on monovalent currents of TRPV6 channels expressed in HEK293 cells.** A and B, time courses of representative whole-cell recordings at a holding potential of −60 mV in HEK293 cells expressing GFP and TRPV6. Monovalent currents were initiated by the addition of NDF solution containing 2 mM EGTA for 20 s followed by addition of 2 mM Ca<sup>2+</sup> or Ba<sup>2+</sup> in NDF for 40 s. Note that the NDF solution at the beginning of the experiment has trace amounts of calcium in the low micromolar range that block monovalent recruitment system (supplemental Fig. S3). Experiments were initiated by the application of a solution containing 2 mM EGTA and no added divalent ions. After a 40-s application of Ca<sup>2+</sup>, but not Ba<sup>2+</sup>, monovalent currents were markedly decreased despite the substantial entry of Ba<sup>2+</sup> observed in fluorescence measurements (see Fig. 2D). The average current amplitude during the first pulse of 0 Ca<sup>2+</sup> (EGTA) was 2.27 ± 0.65 nA, which was significantly higher than the second (0.86 ± 0.38 nA) and third (0.69 ± 0.27 nA) pulses. When Ba<sup>2+</sup> was used instead of Ca<sup>2+</sup>, the average current amplitude for the first pulse was 1.87 ± 0.48 nA. The average current amplitudes for the second and third pulses were 1.83 ± 0.34 and 1.95 ± 0.32 nA, respectively. The same protocol did not induce any current in cells transfected with GFP (Fig. 1, C and F) or non-transfected HEK cells (data not shown). Monovalent currents through TRPV6 are also blocked by extracellular Mg<sup>2+</sup> (11); thus we omitted Mg<sup>2+</sup> from the extracellular NDF solution throughout the experiments.

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**RESULTS**

**Ca<sup>2+</sup>, but Not Ba<sup>2+</sup>, Inactivates Na<sup>+</sup> Currents through TRPV6**—We studied the mechanism of Ca<sup>2+</sup>-induced inactivation of TRPV6 by measuring monovalent currents before and after exposing the TRPV6-expressing cells to Ca<sup>2+</sup> (or Ba<sup>2+</sup>)-containing solutions in whole-cell configuration at a constant holding potential of −60 mV (Fig. 1). Monovalent currents were initiated by the application of a solution containing 2 mM EGTA and no added divalent ions. After a 40-s application of Ca<sup>2+</sup>, but not Ba<sup>2+</sup>, monovalent currents were markedly decreased despite the substantial entry of Ba<sup>2+</sup> observed in fluorescence measurements (see Fig. 2D). The average current amplitude during the first pulse of 0 Ca<sup>2+</sup> (EGTA) was 2.27 ± 0.65 nA, which was significantly higher than the second (0.86 ± 0.38 nA) and third (0.69 ± 0.27 nA) pulses. When Ba<sup>2+</sup> was used instead of Ca<sup>2+</sup>, the average current amplitude for the first pulse was 1.87 ± 0.48 nA. The average current amplitudes for the second and third pulses were 1.83 ± 0.34 and 1.95 ± 0.32 nA, respectively. The same protocol did not induce any current in cells transfected with GFP (Fig. 1, C and F) or non-transfected HEK cells (data not shown). Monovalent currents through TRPV6 are also blocked by extracellular Mg<sup>2+</sup> (11); thus we omitted Mg<sup>2+</sup> from the extracellular NDF solution throughout the experiments.

**Ca<sup>2+</sup>, but Not Ba<sup>2+</sup>, Inactivates Na<sup>+</sup> Currents through TRPV6 Channels Reduces Pl(4,5)P<sub>2</sub> Levels**—Ca<sup>2+</sup> influx through TRPM8 channels has been suggested to induce Pl(4,5)P<sub>2</sub> depletion via PLC activation (23). To explore the mechanism of the differential behavior of Ca<sup>2+</sup> and Ba<sup>2+</sup> on the inactivation of TRPV6 channels, we used a FRET-based technique (23, 33) to show that Ca<sup>2+</sup>, but not Ba<sup>2+</sup>, induces depletion of Pl(4,5)P<sub>2</sub> (Fig. 2, A and B). This technique is based on the translocation of the CFP/YFP-tagged PLC<sub>B1</sub> PH domains from the plasma membrane to the cytoplasm upon PIP(4,5)_{2} depletion, which is shown in the figure as downward deflection of the FRET ratio traces. This technique has been shown to display good correlation with translocation of the GFP-tagged PLC<sub>B1</sub> PH domain as measured with confocal microscopy (33). We also show that Ca<sup>2+</sup> induces translocation of the GFP-tagged PLC<sub>B1</sub> PH domain using confocal microscopy (supplemental Fig. S1). Fig. 2C summarizes the percentage of change in FRET ratio caused by addition of 2 mM Ca<sup>2+</sup> or Ba<sup>2+</sup>. Fig. 2D shows that addition of 2 mM Ca<sup>2+</sup> or Ba<sup>2+</sup> resulted in similar change in the fluorescence ratio of fura-2-
loaded TRPV6 cells, indicating that both Ca\(^{2+}\) and Ba\(^{2+}\) enter through these channels.

We have also measured IP\(_{3}\) production in HEK cells in response to Ca\(^{2+}\) and Ba\(^{2+}\) influx through TRPV6. Fig. 2E shows that IP\(_{3}\) production is increased in HEK cells expressing TRPV6 in response to the application of Ca\(^{2+}\), but not Ba\(^{2+}\). HEK cells not expressing TRPV6 did not respond with increased formation of IP\(_{3}\) to the application of Ca\(^{2+}\) (Fig. 2F), but they responded to extracellular ATP, which activates PLC in these cells via P2Y cell surface receptors (35).

Supplemental Fig. S2 shows that application of extracellular Ca\(^{2+}\) in HEK293 cells not expressing TRPV6 did not significantly increase cytoplasmic Ca\(^{2+}\) levels and did not induce any changes in FRET, demonstrating that Ca\(^{2+}\) entry and Ca\(^{2+}\)-induced PI(4,5)P\(_{2}\) hydrolysis depend on the presence of TRPV6 channels.

Pi(4,5)P\(_{2}\) but Not Pi(4)P, Activates TRPV6 Channels and Prevents Ca\(^{2+}\)-induced Inactivation—We next examined the direct effects of phosphoinositides on TRPV6 channels. We studied the effects of short acyl chain (dC\(_{8}\)) analogues (36) of various phosphoinositides in excised patches of Xenopus oocytes expressing TRPV6 (Fig. 3, A and B). Pi(4,5)P\(_{2}\), but not Pi or Pi(4)P, activated TRPV6 channels in excised patches. Pi(3,4)P\(_{2}\) and Pi(3,4,5)P\(_{3}\), the products of phosphoinositide 3 kinases, also activated TRPV6, but their effects were smaller than that of Pi(4,5)P\(_{2}\) (Fig. 3, A and B).

In whole-cell patch clamp experiments, dialysis of dC\(_{8}\) Pi(4,5)P\(_{2}\) via the patch pipette relieved TRPV6 currents from Ca\(^{2+}\)-induced inactivation (Fig. 4C), whereas dC\(_{8}\) Pi(4)P had no effect (Fig. 4B). Control cells (Fig. 4, A and D) had current amplitudes of 1.44 ± 0.45 (first pulse), 0.49 ± 0.1 (second pulse), and 0.44 ± 0.12 nA (third pulse). The amplitude of the
Translocation of the PI(4,5)P2 5-phosphatase to the plasma membrane inhibits TRPV6. HEK293 cells were transfected with TRPV6 and the CFP-tagged FRB and either the RFP-tagged FKBP12 fused to the phosphatase domain of the PI(4,5)P2 5-phosphatase (5-Phos) or the RFP-tagged FKBP12 (Control). Whole-cell recordings were performed at a constant holding potential of −60 mV in NDF solution. Monovalent currents were initiated with NDF solution containing 2 mM EGTA. Translocation of the phosphatase domain was induced by the addition of 100 nM rapamycin. A and B represent the time course of monovalent currents in TRPV6 cells in a holding potential of −60 mV before and after addition of 100 nM rapamycin in 5-phosphatase and control cells, respectively. C and D represent the mean ± S.E. of current values 30 s after initiation of monovalent current by the addition of 0 Ca2+ (EGTA) solution and 30 s after the addition of rapamycin in 5-phosphatase (n = 9) or control (n = 10) cells, respectively. **, p < 0.001.

FIGURE 4. PI(4,5)P2, but not PI(4)P, prevents Ca2+−induced inactivation of TRPV6 channels in HEK293 cells. A–C represent the time courses of whole-cell TRPV6 currents in control (n = 9), PI(4)P (n = 7), or PI(4,5)P2 (n = 9) dialyzed cells recorded at a constant holding potential of −60 mV. Recordings were performed 5–10 min after the formation of whole-cell configuration. Short acyl chain (DiC8) PI(4)P or PI(4,5)P2 at a concentration of 50 μM were dialyzed through the patch pipette. D–F represent the average current amplitudes ± S.E. normalized to the peak of the first pulse of 0 Ca2+ in control, PI(4)P, and PI(4,5)P2 dialyzed cells, respectively. **, p < 0.001.

FIGURE 5. Translocation of the PI(4,5)P2 5-phosphatase to the plasma membrane inhibits TRPV6. HEK293 cells were transfected with TRPV6 and the CFP-tagged FRB and either the RFP-tagged FKBP12 fused to the phosphatase domain of the PI(4,5)P2 5-phosphatase (5-Phos) or the RFP-tagged FKBP12 (Control). Whole-cell recordings were performed at a constant holding potential of −60 mV in NDF solution. Monovalent currents were initiated with NDF solution containing 2 mM EGTA. Translocation of the phosphatase domain was induced by the addition of 100 nM rapamycin. A and B represent the time course of monovalent currents in TRPV6 cells in a holding potential of −60 mV before and after addition of 100 nM rapamycin in 5-phosphatase and control cells, respectively. C and D represent the mean ± S.E. of current values 30 s after initiation of monovalent current by the addition of 0 Ca2+ (EGTA) solution and 30 s after the addition of rapamycin in 5-phosphatase (n = 9) or control (n = 10) cells, respectively. **, p < 0.001.

monovalent currents in cells dialyzed with DiC8 PI(4)P (Fig. 4, B and E) were 1.65 ± 0.35, 0.48 ± 0.16, and 0.34 ± 0.1 nA for the first, second, and third pulses, respectively. In cells dialyzed with DiC8 PI(4,5)P2 (Fig. 4, C and F), the current amplitude for the first pulse was 1.1 ± 0.14, for the second pulse it was 1.18 ± 0.17, and it was 1.16 ± 0.18 nA for the third pulse.

Dephosphorylation of PI(4,5)P2 by a PI(4,5)P2 5-Phosphatase Inhibits TRPV6 Channels—Next we tested the effect of depletion of PI(4,5)P2 by alternate means that do not involve a rise in intracellular [Ca2+]. For this, we employed the recently developed rapamycin-inducible PI(4,5)P2 5-phosphatase to deplete PI(4,5)P2 in TRPV6-expressing cells (Fig. 5). This technique is based on the translocation of the phosphatase domain of the PI(4,5)P2 5-phosphatase to the plasma membrane induced by rapamycin, which was shown to cause depletion of PI(4,5)P2 and inhibition of TRPM8 (32) and KCNQ2/3 channels (37). Rapamycin (100 nM) inhibited the whole-cell monovalent currents through TRPV6 channels expressing the rapamycin-inducible PI(4,5)P2 5-phosphatase constructs (Fig. 5A) and had no effect in parallel controls (Fig. 5B). Supplemental Fig. S3 shows the translocation of the RFP-tagged PI(4,5)P2 phosphatase to the plasma membrane.

WMN Inhibits TRPV6 Channels—WMN at high concentrations inhibits some isoforms of phosphatidylinositol 4-kinase (38). WMN was reported to deplete PI(4,5)P2 in intact cells and inhibit PI(4,5)P2−sensitive ion channels (39, 40). We found that preincubation with 10 μM WMN for 30 min significantly inhibited TRPV6 currents compared with untreated controls (Fig. 6A–C). We measured the currents at two different time points to compare the peak and the sustained TRPV6 currents in WMN-treated cells and vehicle-treated controls. In control cells (Fig. 6A), the average currents at the end of 10 and 40 s were 1.35 ± 0.27 and 1.34 ± 0.18 nA, respectively, whereas in WMN-treated cells (Fig. 6B) the current remaining at the end of 10 s was 0.098 ± 0.048 nA and at the end of 40 s was 0.063 ± 0.032 nA. Interestingly, WMN-treated cells (four of the six cells) showed slow inactivation of currents even in the absence of extracellular Ca2+. Treatment of cells with 10 μM WMN for a shorter duration of time (10 min) did not result in consistent inhibition of TRPV6 currents (data not shown).

To further confirm the effect of WMN, we measured intracellular Ca2+ in cells expressing TRPV6. Treatment of cells with 10 μM WMN for 30 min inhibited Ca2+ entry significantly compared with untreated controls (Fig. 6D). Fig. 6E summarizes the change in fluorescence ratio in control and WMN-treated cells measured at two different time points of 30 and 150 s after the addition of Ca2+.

Protein Kinase C Activation Does Not Affect TRPV6 Channel Activity—Protein kinase C is also generally activated upon PLC activation, and Ca2+-dependent protein kinase C activation has
been suggested to mediate menthol-induced desensitization of TRPM8 (41, 42). To test the effect of protein kinase C, we examined the effect of 1-oleoyl-2-acetyl-sn-glycerol, a cell-permeable diacylglycerol analogue. 1-Oleoyl-2-acetyl-sn-glycerol (100 μM) failed to inhibit monovalent currents through TRPV6 measured at −60 mV, and it also failed to affect Ca2+ signals in TRPV6-expressing cells (data not shown).

**Direct Application of Ca2+ to Excised Patches Has Only a Negligible Effect on TRPV6**—Finally, we tested the effect of direct application of Ca2+ (10 μM) on TRPV6 in excised patches. These measurements were performed in Xenopus oocytes, which endogenously express a Ca2+-activated Cl− current. We could not fully inhibit these channels with niflumic acid or flufenamic acid (43) at 300 μM. Higher concentrations of these agents inhibited TRPV6 currents (data not shown).

Thus, we circumvented this problem by detecting TRPV6 currents at the reversal potential of the chloride current the following way. For the bath solution (cytoplasmic) we used a gluconate-based Cl−-free solution, and the pipette solution (extracellular) contained Cl− as the main anion (see “Experimental Procedures” for details). The Ca2+-activated Cl− channels have a small but detectable permeability to gluconate (44); thus, we found that the reversal potential of the Cl− (gluconate) currents under these conditions was −103 mV. At this potential we could measure TRPV6 currents, whereas we could monitor the chloride currents at +100 mV (Fig. 7). We applied 10 μM free Ca2+ (buffered with HEDTA) shortly after excision, where it induced only a negligible inhibition of TRPV6 currents (Fig. 7A). As the currents under these conditions exhibited a variable level of rundown (see also Fig. 3), we also applied Ca2+ after the channels were re-activated with 50 μM diC8 PI(4,5)P2 (Fig. 7). Under these conditions 10 μM Ca2+ slightly potentiated TRPV6 currents, but this effect was variable and not statistically significant (p = 0.094, n = 8).

To induce uniform rundown of TRPV6 currents in all experiments, we applied Mg2+ (43 μM free Mg2+) to the excised patches before reactivating TRPV6 with PI(4,5)P2. Mg2+ serves as a cofactor for lipid phosphatases and thus promotes depletion of PI(4,5)P2 (45) that leads to current rundown. Mg2+ also has a direct inhibitory effect on TRPV6 (11), which is mainly prevalent at positive voltages at this concentration; note the fast inhibition of the outward currents in Fig. 7. After the washout of diC8 PI(4,5)P2 when TRPV6 currents completely disappeared, we applied a third pulse of Ca2+ in each measurement to confirm the absence of Cl− current at −103 mV. We conclude that direct binding of Ca2+ to the cytosolic surface of TRPV6 is unlikely to significantly contribute to the marked Ca2+-induced inactivation we observe in whole-cell patch clamp measurements.

**DISCUSSION**

Ca2+–induced Inactivation of TRPV6—TRPV6 is a constitutively active Ca2+-selective channel that mediates Ca2+ uptake through the apical membrane of epithelial cells (4). When Ca2+ enters the cells through these channels, they inactivate, which is mediated by an increase in cytoplasmic [Ca2+]. This Ca2+-induced inactivation may play a role as a feedback loop to regulate cytoplasmic Ca2+ levels, preventing Ca2+ overload through these channels (10). This Ca2+-induced inactivation has been shown to consist of a fast and a slower component when studied by activating the channels with fast voltage steps to negative membrane potentials (12–14). Recovery from this Ca2+-induced inactivation is quite slow (12), suggesting either a process with a very slow off rate or a need for the resynthesis of a cofactor that is lost during the inactivation process.

Most earlier studies examined Ca2+-induced inactivation at relatively high extracellular Ca2+ concentrations (≥10 mM) and on a relatively short time scale (1–2 s), and Ca2+ entry was initiated by a short voltage pulse to negative membrane potentials (12, 13). Our study focused on the effects of steady-state Ca2+ entry at a constant holding potential on a longer time scale (min), as this presumably resembles the native conditions of these channels in epithelial cells. In patch clamp experiments we measured monovalent currents through TRPV6 because these are much easier to detect than the much smaller Ca2+ currents in physiological extracellular Ca2+ concentrations. We show that under these conditions TRPV6 channels undergo
PI(4,5)P₂ depletion is an attractive candidate to mediate the inactivation of TRPV6. We have shown here that dialyzing PI(4,5)P₂ through the patch pipette essentially eliminated the Ca²⁺-induced inactivation of TRPV6. This is compatible with the inactivation being mediated by PI(4,5)P₂ depletion, and it is incompatible with the role of all other candidates, because supplying more substrate for PLC would presumably increase the formation of all the other messengers. Our negative control was PI(4)P, which, unlike PI(4,5)P₂, did not activate TRPV6 in excised patches and did not inhibit Ca²⁺-induced inactivation of TRPV6. We have also shown that activation of protein kinase C with the diacylglycerol analogue 1-oleoyl-2-acetyl-sn-glycerol did not inhibit TRPV6, confirming that Ca²⁺-induced inactivation is not mediated by protein kinase C. We also did not detect substantial inhibition by Ca²⁺ in excised patches; thus, it is unlikely that direct binding of Ca²⁺ to cytoplasmic parts of the channel significantly contributes to Ca²⁺-induced inactivation.

We have shown that PI(4,5)P₂ depletion is necessary for Ca²⁺-induced inactivation of TRPV6, but is it sufficient to inhibit these channels? To test this, we have utilized two different tools to decrease membrane PI(4,5)P₂ levels without activating PLC and thus not forming IP₃ and diacylglycerol. First, we used the recently described rapamycin-inducible PI(4,5)P₂ 5-phosphatase recruitment system to selectively deplete PI(4,5)P₂ by converting it to PI(4)P (32). Rapamycin-induced PI(4,5)P₂ depletion inhibited TRPV6 currents, which is compatible with the role of PI(4,5)P₂ keeping this channel open and the lack of ability of PI(4)P to activate it. To confirm these data, we have also utilized wortmannin at concentrations where it inhibits phosphoinositol 4 kinase thus inhibiting the supply of the precursor of PI(4,5)P₂, leading to slow depletion of PI(4,5)P₂. Wortmannin inhibited both TRPV6 currents and Ca²⁺ signals in TRPV6-expressing cells. These data together demonstrate that depletion of PI(4,5)P₂ is sufficient to inhibit TRPV6.

PI(3,4)P₂ and PI(3,4,5)P₃, the products of phosphatidylinositol 3 kinase, also activated TRPV6 in excised patches even though they were less effective than PI(4,5)P₂. These lipids are thought to be at lower concentrations in the plasma membrane than PI(4,5)P₂ (47), and thus their effects are probably overridden by the latter.

In summary, our data demonstrate that TRPV6 channels require PI(4,5)P₂ for activity and that the hydrolysis of this lipid by Ca²⁺-induced activation of PLC contributes to inactivation of this channel. This mechanism may serve as a feedback loop for the regulation of TRPV6, allowing this channel to function as a Ca²⁺ sensor and thus regulate cytoplasmic Ca²⁺ levels.

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