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\textsuperscript{2+} absorption in the intestines. These channels display high selectivity for Ca\textsuperscript{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\textsuperscript{2+} concentrations. Here we studied the mechanism of this Ca\textsuperscript{2+}-induced inactivation. Monovalent currents through TRPV6 substantially decreased after a forty-second application of Ca\textsuperscript{2+}, but not Ba\textsuperscript{2+}. We also show that Ca\textsuperscript{2+}, but not Ba\textsuperscript{2+} influx via TRPV6 induces depletion of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P\textsubscript{2} or PIP\textsubscript{2}] and the formation of inositol 1,4,5-trisphosphate (IP\textsubscript{3}). Dialysis of DiC\textsubscript{8} PI(4,5)P\textsubscript{2} through the patch pipette inhibited Ca\textsuperscript{2+} dependent inactivation of TRPV6 currents in whole-cell patch clamp experiments. PI(4,5)P\textsubscript{2} also activated TRPV6 currents in excised patches. PI(4)P, the precursor of PI(4,5)P\textsubscript{2} neither activated TRPV6 in excised patches, nor had any effect on Ca\textsuperscript{2+}-induced inactivation in whole-cell experiments. Conversion of PI(4,5)P\textsubscript{2} to PI(4)P by a rapamycin-inducible PI(4,5)P\textsubscript{2} 5-phosphatase inhibited TRPV6 currents in whole-cell experiments. Inhibiting phosphatidylinositol 4-kinases with wortmannin decreased TRPV6 currents and Ca\textsuperscript{2+} entry into TRPV6 expressing cells. We propose that Ca\textsuperscript{2+} influx through TRPV6 activates phospholipase C (PLC) and the resulting depletion of PI(4,5)P\textsubscript{2} contributes to the inactivation of TRPV6.

Calcium signaling orchestrates a myriad of physiological functions such as muscle contraction, neurotransmitter release, bone formation and fertilization. Ca\textsuperscript{2+} entry through plasma membrane ion channels regulates numerous physiological and pathophysiological processes. The essential role of transient receptor potential (TRP) channel proteins in the regulation of cellular Ca\textsuperscript{2+} signaling has begun to be appreciated in the recent past (1-3). The mammalian TRP superfamily comprises of 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\textsuperscript{2+} selectivity (4).

TRPV6 is expressed in Ca\textsuperscript{2+} transporting epithelial cells and it plays an important role in the active Ca\textsuperscript{2+} absorption by the intestines (5;6). TRPV6 channels have been reported to be expressed in a variety of other tissues such as the kidneys, prostate, the stomach, the brain, and the lungs (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\textsuperscript{2+} reabsorption, increased urinary Ca\textsuperscript{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\textsuperscript{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 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)P\(_2\) (16-18). Among the TRP proteins, seven are reported to be modulated by PI(4,5)P\(_2\). The Drosophila TRPL (19) was reported to be inhibited by PI(4,5)P\(_2\), 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)P\(_2\).

In this study we examined the role of phosphoinositides in the Ca\(^{2+}\)-induced inactivation of TRPV6. We demonstrate that the activity of TRPV6 depends on PI(4,5)P\(_2\) using different approaches including the direct activation by PI(4,5)P\(_2\) in excised patches, dialyzing DiC\(_8\) PI(4,5)P\(_2\) via the patch pipette in whole-cell recordings and by dephosphorylating plasma membrane PI(4,5)P\(_2\) using a rapamycin-inducible PI(4,5)P\(_2\) 5-phosphatase. We also show that Ca\(^{2+}\) flowing through TRPV6 activates PLC that leads to the depletion of PI(4,5)P\(_2\). Taken together we provide evidence for a model that envisages the activation of PLC by Ca\(^{2+}\), which results in the hydrolysis of PI(4,5)P\(_2\) causing inactivation of TRPV6 channels.

**EXPERIMENTAL PROCEDURES**

**Cell culture and transfection**

HEK293 cells were maintained in minimal essential medium (MEM) 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 Ca\(^{2+}\) imaging and electrophysiology experiments, transfection was confirmed by measuring 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 PIP\(_2\) 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 hours 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 Ca\(^{2+}\) imaging. Borosilicate glass pipettes (World Precision Instruments) of 2-4 mega ohm-resistance were filled with a solution containing, in mM, 135 K-Gluconate, 5 KCl, 5 EGTA, 1 MgCl\(_2\), 2 ATP disodium, 10 HEPES, pH adjusted to 7.2. The cells were kept in NDF solution for 20 minutes before measurements. After formation of gigaohm resistance seals, whole-cell configuration was established and currents were measured using an Axopatch 200B amplifier (Axon Instruments). Data were collected and analyzed with pCLAMP 9.0.
Software. All measurements were performed at room temperature 20-25 °C. For experiments with wortmannin (WMN), cells expressing TRPV6 were preincubated with NDF containing 10 μM WMN for 30 minutes at room temperature. Control cells were treated with vehicle (DMSO) in NDF. All conditions and solutions remain the same for patch clamp, Ca²⁺ imaging and FRET experiments unless otherwise specified.

Oocyte excised patch experiments
Macropatch experiments were performed with 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 LiCl, 1 EGTA and 5 HEPES, pH 7.4. For the measurements 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 K gluconate, 5 HEDTA, 5 HEPES, pH 7.4. The free Ca²⁺ and Mg²⁺ concentrations were calculated using the MaxChelator program http://www.stanford.edu/~cpatton/maxc.html. To result in 10 μM free calcium, 3.85 mM Ca²⁺ was added (Ca-gluconate) and to result in 43 μM free Mg²⁺, we added 2.35 mM Mg²⁺ (Mg-gluconate). For these measurements the bath was connected with the ground electrode through an agar bridge. Data were analyzed with the pCLAMP 9.0 software (Axon Instruments), and plotted using Microcal Origin.

Fluorescence Resonance Energy Transfer (FRET) measurements
HEK293 cells were 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, 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), 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 reference (34). HEK293 cells were transfected with either TRPV6-myc and GFP or with GFP alone (controls) and incubated with 20 μCi of ³H-myoinositol overnight in growth medium. Before the experiments the cells were kept in NDF for 20 minutes, and an additional 10 minutes 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 minutes in the continued presence of LiCl. The cells were then scraped, treated with 4% perchloric acid and centrifuged at 12000 rpm for 2-4 minutes. The supernatant (1.2 ml) was transferred into glass tubes containing 180 μl of 10 mM EDTA (pH 7.0) and to each tubes 1.3 ml of freshly prepared mixture of trioctylamine/Freon mixture was added, vortexed and centrifuged at 12000 rpm for 4 minutes. The top aqueous layer (approximately 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 AG1x8 resin (formate form). The columns were then washed 4 times each with 5 ml of distilled water. Fractions 1-4 (5 ml each) 0.4 M ammonium formate/0.1M formic acid pH 4.75 (IP₂ fraction), fractions 5-8 (5 ml each) 0.7 M ammonium formate/0.1M formic acid pH 4.75 (IP₃ fraction) and fractions 9-12 (5 ml each) 1.2 M ammonium formate/0.1 M formic acid pH 4.75 (IP₄, IP₅ and IP₆ fraction) were collected. One ml of each of the collected fraction was transferred into a scintillation vial, 10 ml of scintillation cocktail was added.
and $^3$H activity was determined in a scintillation counter.

**Ca$^{2+}$ imaging**

Cells transfected with TRPV6 and GFP (marker for cell selection) were grown on 25 mm circular coverslips and loaded with fura-2 AM (2 µM) for 30–40 min at room temperature in NDF. The coverslips were then washed in NDF, placed in a stainless-steel holder (bath volume, ~0.8 ml; Molecular Probes), and viewed in a Zeiss Axiovert 100 microscope coupled to an Attofluor digital imaging system. Cells expressing GFP were selected and monitored simultaneously on each coverslip. Results are presented as the ratio ($R$) of fluorescence intensities at excitation wavelengths of 334 nm and 380 nm. Cells were continuously superfused with NDF and Ca$^{2+}$ entry was initiated by addition of a solution containing 2 mM CaCl$_2$. All experiments were performed at room temperature.

**Confocal microscopy**

HEK293 cells were transfected with TRPV6 and the GFP-tagged PLC$\delta_1$ PH-domain (Supplemental Fig. 1), or the components of the PI(4,5)P$_2$ phosphatase recruitment system (Supplemental Fig. 3). Experiments were performed two days after transfection. The cells were observed with a Zeiss LSM-510 confocal microscope in the Confocal Imaging Facility of the New Jersey Medical School. Images were saved as TIFF files and were analyzed with the IMAGE J software.

**Materials**

Fura-2 AM was obtained from TefLabs (Austin, Texas). Effectene was obtained from Qiagen. Cell culture media, antibiotics and sera were obtained from Invitrogen. DiC$_3$ phosphoinositides were obtained from Cayman and Echelon. All other chemicals were purchased from Sigma.

**Data analysis**

Data for all figures were expressed as mean ± S.E.M. Statistical significance was evaluated by student t-test, * stands for p<0.05 and ** for p<0.001.

**RESULTS**

Ca$^{2+}$ but not Ba$^{2+}$ inactivates Na$^+$ currents through TRPV6

We studied the mechanism of Ca$^{2+}$-induced inactivation of TRPV6 by measuring monovalent currents before and after exposing the TRPV6 expressing cells to Ca$^{2+}$ (or Ba$^{2+}$) 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 forty-second application of Ca$^{2+}$ but not Ba$^{2+}$ monovalent currents were markedly decreased despite the substantial entry of Ba$^{2+}$ observed in fluorescence measurements (see Fig. 2D). The average current amplitude during the first pulse of 0 Ca$^{2+}$ (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$^{2+}$ was used instead of Ca$^{2+}$, 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 nA and 1.95 ± 0.32 respectively. The same protocol did not induce any current in cell 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$^{2+}$ (11) thus we omitted Mg$^{2+}$ from extracellular NDF solution throughout the experiments.

Ca$^{2+}$ but not Ba$^{2+}$ influx through TRPV6 channels reduces PIP$_2$ levels

Ca$^{2+}$ influx through TRPM8 channels has been suggested to induce PI(4,5)P$_2$ depletion via PLC activation (23). In order to explore the mechanism of the differential behavior of Ca$^{2+}$ and Ba$^{2+}$ on the inactivation of TRPV6 channels, we used a fluorescence resonance energy transfer (FRET) based technique (23;33) to show that Ca$^{2+}$ but not Ba$^{2+}$ induces depletion of PI(4,5)P$_2$ (Fig. 2A, 2B). The technique is based on the translocation of the CFP/YFP tagged PLC$\delta_1$ PH domain from the plasma membrane to the cytoplasm upon PI(4,5)P$_2$ 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 PLCo1 PH domain as measured with confocal microscopy (33). We also show that Ca$^{2+}$ induces translocation of the GFP-tagged PLCδ1 PH domain using confocal microscopy (Supplemental Fig. 1). Fig. 2C summarizes the percentage of change in FRET ratio caused by addition of 2 mM Ca$^{2+}$ or Ba$^{2+}$. Fig. 2D shows that addition of 2 mM Ca$^{2+}$ or Ba$^{2+}$ resulted in similar change in the fluorescence ratio of fura-2AM loaded TRPV6 cells, indicating that both Ca$^{2+}$ and Ba$^{2+}$ enters 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. 2 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 IP$_3$ 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 (diC$_8$) analogues (36) of various phosphoinositides in excised patches of Xenopus oocytes expressing TRPV6 (Fig. 3A 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 PI3K also activated TRPV6, but their effects were smaller than that of PI(4,5)P$_2$ (Fig. 3A and B).

In whole-cell patch clamp experiments, dialysis of diC$_8$ PI(4,5)P$_2$ via the patch pipette relieved TRPV6 currents from Ca$^{2+}$-induced inactivation (Fig. 4C), whereas diC$_8$ PI(4)P had no effect (Fig. 4B). Control cells (Fig. 4A,D) had current amplitudes of 1.44 ± 0.45 nA (first pulse), 0.49 ± 0.1 nA (second pulse) and 0.44 ± 0.12 nA (third pulse). The amplitude of the monovalent currents in cells dialyzed with diC$_8$ PI(4)P (Fig. 4B,E) were 1.65 ± 0.35 nA, 0.48 ± 0.16 nA and 0.34 ± 0.1 nA for the first, second and third pulses, respectively. In cells dialyzed with diC$_8$ PI(4,5)P$_2$ (Fig. 4C,F), the current amplitude for the first pulse was 1.1 ± 0.14 nA, for the second pulse it was 1.18 ± 0.17 nA and it was 1.16 ± 0.18 nA for the third pulse.

**Dephosphorylation of PI(4,5)P$_2$ by a PI(4,5)P$_2$ 5-phosphatase inhibits TRPV6 channels** Next we tested the effect of depletion of PI(4,5)P$_2$ by alternate means that does not involve a rise in intracellular [Ca$^{2+}$]. For this, we employed the recently developed rapamycin-inducible PI(4,5)P$_2$ 5-phosphatase to deplete PI(4,5)P$_2$ in TRPV6 expressing cells (Fig. 5). This technique is based on the translocation of the phosphatase domain of the PI(4,5)P$_2$ 5-phosphatase to the plasma membrane induced by rapamycin, which was shown to cause depletion of PI(4,5)P$_2$ 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)P$_2$ 5-phosphatase constructs (Fig. 5A) and had no effects in parallel controls (Fig. 5B). Supplemental Fig. 3 shows the translocation of the RFP tagged PI(4,5)P$_2$ phosphatase to the plasma membrane.

**Wortmannin (WMN) inhibits TRPV6 channels** WMN, at high concentrations inhibits some isoforms of phosphatidylinositol 4-kinase (PI4K) (38). WMN was reported to deplete PI(4,5)P$_2$ in intact cells and inhibit PI(4,5)P$_2$ sensitive ion channels (39;40). We found that pre-incubation with 10 μM WMN for 30 minutes significantly inhibited TRPV6 currents compared to 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 current at the end of 10 seconds and 40 seconds were 1.35 ± 0.27 nA and 1.34 ± 0.18 nA respectively, while in WMN treated cells (Fig. 6B) the
current remaining at the end of 10 seconds was 0.098 ± 0.048 nA and at the end of 40 seconds was 0.063 ± 0.032 nA. Interestingly, WMN treated cells (4 out of the 6 cells) showed slow inactivation of currents even in the absence of extracellular Ca²⁺. Treatment of cells with 10 µM WMN for shorter duration of time (10 minutes) 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 minutes inhibited Ca²⁺ entry significantly compared to 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 seconds and 150 seconds after the addition of Ca²⁺.

**Protein Kinase C (PKC) activation does not affect TRPV6 channel activity** PKC is also generally activated upon PLC activation, and Ca²⁺-dependent PKC activation has been suggested to mediate menthol-induced desensitization of TRPM8 (41;42). To test the effect of PKC, we examined the effect of OAG, a cell permeable DAG analogue. OAG (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 only has a negligible effect on TRPV6.** Finally we tested the effects 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 methods 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 this condition 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 co-factor for lipid phosphatases thus promotes depletion of PI(4,5)P₂ (45) that leads to current rundown. Mg²⁺ also have a direct inhibitory effect on TRPV6 (Voets et al JGP 2003), 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 feed back loop to regulate cytoplasmic Ca²⁺ levels preventing Ca²⁺ overload through these
channels (10). This Ca\(^{2+}\)-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 Ca\(^{2+}\)-induced inactivation is quite slow (12), suggesting either a process with a very slow off rate, or a need for the resynthesis of a co-factor that is lost during the inactivation process.

Most earlier studies examined Ca\(^{2+}\)-induced inactivation at relatively high extracellular [Ca\(^{2+}\)] \((\geq 10 \text{ mM})\), and on a relatively short time scale (1-2 seconds), and Ca\(^{2+}\) entry was initiated by a short voltage pulse to negative membrane potentials (12,13). Our study focused on the effects of steady state Ca\(^{2+}\) entry at a constant holding potential, on a longer time scale (minutes), 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 Ca\(^{2+}\) currents in physiological extracellular Ca\(^{2+}\) concentrations. We show that under these conditions TRPV6 channels undergo inactivation when conducting Ca\(^{2+}\), but not Ba\(^{2+}\), which is consistent with earlier findings using different protocols (14).

We show that PI(4,5)P\(_2\) is depleted in response to application of extracellular Ca\(^{2+}\) but not Ba\(^{2+}\) in cells expressing TRPV6. We also show that Ca\(^{2+}\), but not Ba\(^{2+}\) stimulates the formation of IP\(_3\) in TRPV6 expressing cells, thus it is likely that the mechanism of PI(4,5)P\(_2\) depletion is the Ca\(^{2+}\)-induced activation of PLC. The lack of effect of Ba\(^{2+}\) on PI(4,5)P\(_2\) depletion and IP\(_3\) formation is consistent with earlier findings showing that PLC activation, as measured by the sustained phase of IP\(_3\) production, was significantly reduced in angiotensin-2 stimulated adrenal glomerulosa cells when Ca\(^{2+}\) was replaced with Ba\(^{2+}\) in the extracellular medium (46).

The role of phosphoinositides in the regulation of TRPV6. The activation of PLC leads to a multitude of events, such as formation of IP\(_3\) and other soluble inositol phosphates, Ca\(^{2+}\) release from intracellular stores, the activation of PKC, and reduction of plasma membrane PI(4,5)P\(_2\) levels. Many TRP channels need PI(4,5)P\(_2\) for activity, thus PI(4,5)P\(_2\) depletion is an attractive candidate to mediate the inactivation of TRPV6. We have shown here that dialyzing PI(4,5)P\(_2\) through the patch pipette essentially eliminated the Ca\(^{2+}\)-induced inactivation of TRPV6. This is compatible with the inactivation being mediated by PI(4,5)P\(_2\) 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)P\(_2\), did not activate TRPV6 in excised patches, and did not inhibit Ca\(^{2+}\)-induced inactivation of TRPV6. We have also shown that activation of PKC with the DAG analogue OAG did not inhibit TRPV6, confirming that Ca\(^{2+}\)-induced inactivation is not mediated by PKC. We also did not detect substantial inhibition by Ca\(^{2+}\) in excised patches, thus it is unlikely that direct binding of Ca\(^{2+}\) to cytoplasmic parts of the channel significantly contribute to Ca\(^{2+}\) induced inactivation.

We have shown that PI(4,5)P\(_2\) depletion is necessary for Ca\(^{2+}\)-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)P\(_2\) levels without activating PLC and thus not forming IP\(_3\) and DAG. First we used the recently described rapamycin-inducible PI(4,5)P\(_2\) 5-phosphatase recruitment system to selectively deplete PI(4,5)P\(_2\) by converting it to PI(4)P (32). Rapamycin-induced PI(4,5)P\(_2\) depletion inhibited TRPV6 currents, which is compatible with the role of PI(4,5)P\(_2\) 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 PI4K, thus inhibiting the supply of the precursor of PI(4,5)P\(_2\) leading to slow depletion of PI(4,5)P\(_2\). Wortmannin inhibited both TRPV6 currents and Ca\(^{2+}\) signals in TRPV6 expressing cells. These data together demonstrate that depletion of PI(4,5)P\(_2\) is sufficient to inhibit TRPV6.
PI(3,4)P₂ and PI(3,4,5)P₃, the products of PI3K 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) 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 feed-back loop for the regulation of TRPV6 allowing this channel to function as a Ca²⁺ sensor, thus regulate cytoplasmic Ca²⁺ levels.

FIGURE LEGENDS

Fig. 1 Effects of Ca²⁺ and Ba²⁺ 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 seconds followed by addition of 2 mM Ca²⁺ or Ba²⁺ 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 blocks monovalent currents through TRPV6. In the figures 1, 2 and 3 represent three alternating pulses of 0 Ca²⁺ (EGTA) and 2 mM Ca²⁺ or Ba²⁺. C representative current trace in HEK cells expressing GFP but not TRPV6. D and E represent the normalized average current amplitudes ± S.E.M remaining before the addition of Ca²⁺ (n=8) or Ba²⁺ (n=8) normalized to the first pulse in TRPV6 expressing cells. F, average current amplitudes evoked by pulses of EGTA in HEK cells expressing GFP alone.

Fig. 2 Ca²⁺ but not Ba²⁺ induces PI(4,5)P₂ hydrolysis in TRPV6 expressing cells. Fluorescence was measured in HEK293 cells expressing TRPV6 and the CFP and YFP tagged PLCδ₁ PH domains as described in the methods section. Cells were kept in NDF solution for 20 minutes before the experiment. During the measurement, 2 mM Ca²⁺ or Ba²⁺ was added at 200 s. A and B represent the time courses of FRET ratio for Ca²⁺ and Ba²⁺ respectively. C represents the mean ± S.E.M for change in FRET ratio signals calculated by dividing the value 2 minutes after the addition of 2 mM Ca²⁺ (n=5) or Ba²⁺ (n=5) by the value before addition to results in F, and then subjecting F in the equation 100-(100 x F). D represents the mean of change in 340/380 nm ratio ± S.E.M for Ca²⁺ (n=31) or Ba²⁺ (n=36) in fura-2 loaded cells expressing TRPV6. E IP₃ measurement in TRPV6 expressing HEK-293 cells in response to 2 mM Ca²⁺ or Ba²⁺. F IP₃ measurement in non-transfected HEK-293 cells, in response to 2 mM Ca²⁺ and in response to 100 μM extracellular ATP.

Fig. 3 PI(4,5)P₂ activates TRPV6 in excised patches Currents were measured in large membrane patches excised from Xenopus oocytes expressing TRPV6 using the ramp protocol from -100 to +100 mV applied every second (0.25 mV/ms). Representative traces show currents at +100 and -100 mV, upper and lower traces, respectively. Phosphoinositides were applied directly to the intracellular surface of the patch, as indicated by the horizontal bars. A. Representative trace describing the effects of various phosphoinositides. To facilitate run-down, 30 μg/ml Poly-Lysine was added before the application of the phosphoinositides in 2 out of 6 experiments; in the remaining experiments phosphoinositides were applied after spontaneous run-down of the currents. The effects of phosphoinositides were indistinguishable in poly-Lysine treated and untreated patches thus the results were pooled. B illustrates statistics for -100 mV (n=6) of the effects of different phosphoinositides compared to PI(4,5)P₂. The average current ± S.E.M for
various phosphoinositides was normalized to PI(4,5)P\textsubscript{2} responses. All phosphoinositides were short acyl-chain (DiC\textsubscript{8}) applied at a concentration of 50 \( \mu \)M.

**Fig. 4** PI(4,5)P\textsubscript{2} but not PI(4)P prevents Ca\textsuperscript{2+}-induced inactivation of TRPV6 channels in HEK293 cells

A, B and C represent the time courses of whole-cell TRPV6 currents in control (n=9), PI(4)P (n=7) or PI(4,5)P\textsubscript{2} (n=9) dialyzed cells recorded at a constant holding potential of -60 mV. Recordings were performed 5 to 10 minutes after the formation of whole-cell configuration. Short acyl-chain (DiC\textsubscript{8}) PI(4)P or PI(4,5)P\textsubscript{2} at a concentration of 50 \( \mu \)M were dialyzed through the patch pipette. D,E, and F represent the average current amplitudes ± S.E.M normalized to the peak of the first pulse of 0 Ca\textsuperscript{2+} in control, PI(4)P and PI(4,5)P\textsubscript{2} dialyzed cells, respectively.

**Fig. 5** Translocation of the PI(4,5)P\textsubscript{2} 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 PIP\textsubscript{2} 5-phosphatase (5'-ptase) 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 at 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.M of current values 30 s after initiation of monovalent current by the addition of 0 Ca\textsuperscript{2+} (EGTA) solution and 30 seconds after the addition of rapamycin in 5-phosphatase (n=9) or control (n=10) cells respectively.

**Fig. 6** Wortmannin (WMN) inhibits monovalent currents as well as Ca\textsuperscript{2+} entry through TRPV6 channels

A and B represent the time courses of whole-cell TRPV6 currents measured in control and WMN pretreated (10 \( \mu \)M for 30 minutes) cells at a constant holding potential of -60 mV. Normalized whole-cell monovalent currents are shown in C for control and WMN pretreated cells. The mean currents ± S.E.M were measured at two time points (1 and 2) and represented for the control (n=8) and WMN pretreated (n=6) cells. D represents the time courses of changes in the ratio of fura-2 fluorescence upon addition of 2 mM Ca\textsuperscript{2+} in WMN pretreated cells and untreated controls. The average change in fluorescence ratio ± S.E.M for the control (n=32) and WMN pretreated (n=66) cells measured at two time points 1 and 2 representing 30 s and 150 s after initiation of Ca\textsuperscript{2+} entry were plotted.

**Fig. 7.** The effect of Ca\textsuperscript{2+} on TRPV6 in excised patches.

A. Macroscopic currents were measured in excised patches from Xenopus oocytes expressing TRPV6, using the solutions described in the methods section. The applications of 10 \( \mu \)M free Ca\textsuperscript{2+}, 43 \( \mu \)M free Mg\textsuperscript{2+} and 50 \( \mu \)M diC\textsubscript{8} PI(4,5)P\textsubscript{2} were indicated with horizontal lines. Holding potential was 0 mV, then a 150 ms long steps to -103 mV followed by a step to +100 mV were applied once every second. The changes at +100 mV mainly correspond to the Ca\textsuperscript{2+} activated Cl\textsuperscript{-} currents (I\textsubscript{Cl-}) whereas the changes at -103 mV correspond exclusively to TRPV6 currents. Note the lack of any current in response to Ca\textsuperscript{2+} at -103 mV after TRPV6 current rundown at the end of the experiment (third pulse of Ca\textsuperscript{2+}).

**FOOTNOTES**

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Abbreviations:
ATP, adenosine 5′-trisphosphate; CFP, cyan fluorescence protein; DAG, diacylglycerol; DMSO, dimethyl sulfoxide; EGTA, (ethylenebis(oxyethylenenitrilo)) tetra-; ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetra acetic acid; HEDTA: N-(2-Hydroxyethyl)ethylenediamine-N,N',N′-triacetic acid; FKBP, FK506 binding protein; FRB, FKBP-12 rapamycin binding protein; FRET, Fluorescence Resonance Energy Transfer; GFP, green fluorescence protein; I, current; IP₃, inositol 1,4,5-trisphosphate; RFP, red fluorescence protein; nA, nano ampere; NDF, nominally divalent free; OAG, 1-oleoyl-2-acetyl-sn-glycerol; PI3K, phosphoinositol 3 kinase; PI(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PI(4)P, phosphatidylinositol 4-monophosphate, PI(3,4,5)P₃, phosphatidylinositol 3,4,5-trisphosphate; PI(3,4)P₂, phosphatidylinositol 3,4-bisphosphate; PLC, phospholipase C; s, second; TRPV6, transient receptor potential protein vanilloid subfamily 6; WMN, wortmannin; PKC, Protein Kinase C; YFP, yellow fluorescent protein; FKBP, FK506 rapamycin binding protein

Reference List

Figure 1

A. HEK-293 TRPV6

B. HEK-293 TRPV6

C. HEK-293 GFP

D. HEK-293 TRPV6

E. HEK-293 TRPV6

F. HEK-293 GFP
Figure 2

A. NDF with 2 mM Ca^{2+} vs. 2 mM Ba^{2+}

B. NDF with 2 mM Ba^{2+} vs. 2 mM Ca^{2+}

C. Change in FRET ratio (%)

D. Change in ratio (340/380)

E. IP_{3} recovery (CPM) for HEK-TRPV6

F. IP_{3} recovery (CPM) for HEK
Figure 3

A

![Graph showing time course of PI(3,4,5)P$_3$ and PI(4,5)P$_2$ with Poly-Lys stimulation.]

B

![Bar graph showing normalized current for different phosphatidylinositols.]

Normalized current

PK(4,5)P$_2$, PI, PI(3,4)P$_2$, PI(3,4,5)P$_3$
Figure 4

A control

B PI(4)P

C PI(4,5)P_2

D

E

F

Normalized current

Pulse 1 | Pulse 2 | Pulse 3

Pulse 1 | Pulse 2 | Pulse 3

Pulse 1 | Pulse 2 | Pulse 3
Figure 5

A: 5-Ptase

B: Control

NDF  EGTA  RAPAMYCIN

I (nA)

Time (s)

Before Rapamycin

After Rapamycin

I (nA)

Before Rapamycin

After Rapamycin
Figure 6