Ca²⁺-Dependent Desensitization of TRPV2 Channels Is Mediated by Hydrolysis of Phosphatidylinositol 4,5-Bisphosphate

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TRPV2 is a member of the transient receptor potential family of ion channels involved in chemical and thermal pain transduction. Unlike the related TRPV1 channel, TRPV2 does not appear to bind either calmodulin or ATP in its N-terminal ankyrin repeat domain. In addition, it does not contain a calmodulin-binding site in the distal C-terminal region, as has been proposed for TRPV1. We have found that TRPV2 channels transiently expressed in F-11 cells undergo Ca²⁺-dependent desensitization, similar to the other TRPVs, suggesting that the mechanism of desensitization may be conserved in the subfamily of TRPV channels. TRPV2 desensitization was not altered in whole-cell recordings in the presence of calmodulin inhibitors or on coexpression of mutant calmodulin but was sensitive to changes in membrane phosphatidylinositol 4,5-bisphosphate (PIP₂), suggesting a role of membrane PIP₂ in TRPV2 desensitization. Simultaneous confocal imaging and electrophysiological recording of cells expressing TRPV2 and a fluorescent PIP₂-binding probe demonstrated that TRPV2 desensitization was concomitant with depletion of PIP₂. We conclude that the decrease in PIP₂ levels on channel activation underlies a major component of Ca²⁺-dependent desensitization of TRPV2 and may play a similar role in other TRP channels.

Introduction

Desensitization of sensory neurons is particularly important considering their crucial role in the physiological perception of and reaction to the external environment. A remarkable example of this type of mechanism is the capsaicin sensitivity of peripheral nociceptors mediated by TRPV1. Topical application of capsaicin to skin causes desensitization of TRPV1 channels expressed in these neurons rendering them less responsive to noxious stimuli (Szallasi and Blumberg, 1999). It is, therefore, through TRPV1 desensitization that peripheral neurons can adapt to painful thermal and chemical stimuli.

TRPV1, as well as many other members of the TRP family, shows a Ca²⁺-dependent desensitization, inhibition, or inactivation (Zhu and Birnbaumer, 1998; Clapham, 2002). This conservation of function is remarkable, suggesting a common molecular mechanism for desensitization among TRP receptors. Despite the strikingly similar desensitization among members in the TRP superfamily and the importance of this form of regulation, recent studies (Juvin et al., 2007; Phelps et al., 2010) have suggested that

TRPV2, a channel closely related to TRPV1, does not exhibit desensitization. Consistent with this idea, a series of molecular studies have shown that TRPV2 does not share the regulatory domains suggested to be important for desensitization of other TRPV channels (Fig. 1). For example, it has been reported that Ca²⁺/calmodulin (Ca²⁺/CaM), an ubiquitous calcium sensor, may play a role in TRPV1 Ca²⁺-dependent desensitization by (1) binding to the N-terminal ankyrin repeat domain (ARD) (Rosenbaum et al., 2004), (2) competing with ATP for a nucleotide-binding site in the ARD (Lishko et al., 2007), and (3) directly binding to a distal C-terminal region (Numazaki et al., 2003) (Fig. 1). In contrast, biochemical studies suggest that the TRPV2 ARD does not appear to bind either CaM or ATP (Lishko et al., 2007; Phelps et al., 2010). Furthermore, the TRPV2 C-terminal region does not show conservation of the proposed TRPV1 C-terminal CaM-binding site (Fig. 1). It has also been shown that Ca²⁺ influx through TRPV1 causes depletion of phosphatidylinositol 4,5-bisphosphate (PIP₂) during desensitization, and the recovery of the channel from desensitization requires resynthesis of the lipid (Liu et al., 2005; Klein et al., 2008). A proximal and distal C-terminal region have been proposed to mediate PIP₂ binding in TRPV1 (Prescott and Julius, 2003; Brauchi et al., 2007), but homologous PIP₂-binding sites in TRPV2 are either absent (distal) or have not been studied (proximal) (Fig. 1).

Here, we report that TRPV2 undergoes Ca²⁺-dependent desensitization similar to TRPV1. This makes TRPV2 an ideal experimental model to study the molecular mechanism of Ca²⁺-dependent desensitization since it does not contain the binding sites for CaM, ATP, or PIP₂ but still shows the prototypical Ca²⁺-dependent desensitization. We show that, although CaM binds *in*

Received April 23, 2010; revised Aug. 6, 2010; accepted Aug. 11, 2010.

This work was supported by National Institutes of Health (NIH) Grant R01EY017564 (S.E.G.), NIH Grant 2R01EY010329-16 (W.N.Z.), and National Eye Institute Core Grant for Vision Research P30EY01730. This work was also supported by the University of Washington Vision Training Grant ST32EY007031-33 (J.M.) and the Howard Hughes Medical Institute. We thank David Julius (University of California, San Francisco, San Francisco, CA) for providing the TRPV2 cDNA, Dr. Mark Lemmon for providing the PLCδ1-PH domain cDNA, and Dr. John Addelman for providing the CaM₁₂₃₄ cDNA. We thank Mika Munari for her excellent technical assistance and Drs. Luis Fernando Santana and Manuel Navedo for technical assistance in the combined imaging and electrophysiology experiments.

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DOI:10.1523/JNEUROSCI.2108-10.2010

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rTRPV2

rTRPV1

Figure 1. Diagram of rat TRPV2 and rat TRPV1 primary sequence. The transmembrane domains (S1–S6) are shown in gray and the pore lining domain is shown in white. The N- and C-terminal regions are cytosolic. TRPV2 and TRPV1 ARDs (Jin et al., 2006; Lishko et al., 2007) are shown in blue, regions suggested to interact with CaM (Numazaki et al., 2003; Rosenbaum et al., 2004) are shown in green, and region suggested to interact with ATP (Kwak et al., 2000; Lishko et al., 2007) is shown in red. Regions in the C-terminal domain of TRPV1 suggested to be important for PIP₂ modulation (proximal residues 686–752 and distal residues 777–820) (Prescott and Julius, 2003; Brauchi et al., 2007) and a potential PIP₂ binding site in the C-terminal domain (proximal residues 647–715) of TRPV2 are shown in orange.

in vitro to a novel binding site in a TRPV2 C-terminal fragment in a Ca²⁺-dependent manner, CaM binding may not be functionally coupled to TRPV2 desensitization. In contrast, we determined that decreases in membrane PIP₂ levels on channel activation underlie a major component of the Ca²⁺-dependent desensitization observed in TRPV2 channels. We propose that decreases in membrane PIP₂ levels are responsible for much, if not all, the Ca²⁺-dependent desensitization seen in TRPV2.

Materials and Methods

Cell culture and transfection. F-11 and human embryonic kidney tsA201-cells were cultured and maintained as previously described (Klein et al., 2008). Cells were transiently transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions and used for electrophysiology 1–4 d after transfection.

Electrophysiology. Whole-cell patch-clamp measurements were obtained at a continuous holding potential of −60 mV. After obtaining stable whole-cell recordings, negative holding pressure was applied, and the cell was lifted from the coverslip and positioned in front of the perfusion pipette. Access resistance was compensated >80% using amplifier circuitry. Whole-cell capacitance values were obtained from the amplifier settings. Solutions were applied using a “sewer pipe” solution changer controlled by RSC-200 (Bio-Logic). The extracellular solution consisted of Hanks buffered salt solution containing the following (in mM): 140 NaCl, 4 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 HEPES, 5 glucose, pH 7.4. For Ca²⁺-free solution measurements, the extracellular solution contained the following (in mM): 145 NaCl, 5 KCl, 1.5 MgCl₂, 1 EGTA, 10 HEPES, 10 glucose, pH 7.4.

Recording electrodes (1–4 MΩ) were fabricated from filamented borosilicate glass pipettes (Sutter Instrument) using a multistage puller (Flaming-Brown model P-97; Sutter Instrument). Recording pipettes were filled with the following (in mM): 110 K-aspartate, 30 KCl, 10 NaCl, 1 MgCl₂, 0.050 EGTA, 10 HEPES, 3 Mg-ATP, 0.1 GTP, pH 7.2. EGTA was replaced with 10 mM 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid (BAPTA) for Ca²⁺-free solution measurements.

For the excised patch experiments, inside-out configuration was established and the patch was positioned in front of the application pipette. Patch pipettes (2–4 MΩ) were filled with symmetrical recording solutions (in mM: 130 NaCl, 3 HEPES, and 0.2 EDTA). As discussed in Re-

sults, we found that TRPV2 currents in inside-out patches underwent an activity-dependent rundown. As expected, this rundown was exacerbated during potentiation of TRPV2 currents (see Fig. 8A) (application of PIP₂), making it difficult to obtain maximal values of activation/modulation. Furthermore, because incorporation of natural PIP₂ into patch membranes was slower than incorporation of diC8-PIP₂, more rundown was apparent in the former case. This phenomenon likely explains the small, not statistically significant difference between the effects of diC8-PIP₂ and natural PIP₂. All currents were recorded with an Axopatch 200B amplifier (Molecular Devices) interfaced to a computer.

Cloning of expression vectors. cDNA fragments encoding the ankyrin repeat domain of rat TRPV1 (residues 147–266), rat TRPV2 (residues 116–192), mouse TRPV3 (residues 163–264), rat TRPV4 (residues 184–287), human TRPV5 (residues 74–166), and human TRPV6 (residues 74–166) were cloned into the EcoRI and XhoI sites of pGEX-6P-1 with the addition of a FLAG epitope to the C terminus. For the TRPV2 C-terminal construct lacking the last seven residues, rat TRPV2 cDNA encoding residues 645–746 were cloned into NcoI and HindIII sites of pHmalC2T and pNGFP_BC (kindly provided by Eric Gouaux, Oregon Health and Science University, Portland, OR) vectors. Proximal (residues 645–684) and distal (residues 704–753) TRPV2 C-terminal constructs were cloned into NcoI and HindIII sites of pNGFP_BC. All clones were verified by DNA sequencing.

Fusion protein expression and purification. Bacteria [BL21 (DE3)] were either transformed or cotransformed with the indicated constructs. Overnight starter cultures were used to inoculate cultures at 1:100; these were grown at 37°C until they reached OD₆₀₀ = 0.6–0.8. IPTG (1 mM) was then added and induction proceeded overnight at 18°C.

After pelleting of bacteria, cells were resuspended in solutions containing the following (in mM): 150 KCl, 1 or 10 CaCl₂, and/or 10 EGTA as indicated, 30 Tris, pH 8.5, and 0.5 tris(2-carboxyethyl)phosphine (TCEP). Large-scale bacterial cultures were lysed by two passages through an Avestin C-5 instrument, whereas small-scale cultures were lysed with a probe sonicator set to 50% duty cycle and power output of 0.5. Lysates were spun at 40,000 rpm for 45 min at 4°C in a Ti-45 ultracentrifuge rotor.

Glutathione S-transferase (GST) fusion proteins were purified using a batch method by incubation with 800 ml of a 50% glutathione (GSH)-agarose slurry for 30 min at room temperature, and then washed extensively with PBS. The proteins were eluted with the following (in mM): 50 Tris, pH 8, 15 GSH, 0.1% Triton X-100 for 30 min. For affinity purification of MBP fusion proteins, a 100 ml column was packed with amylose resin (New England BioLabs) and equilibrated with solution A (in mM: 150 KCl, 10 CaCl₂, 30 Tris, pH 8.5, and 0.5 TCEP). The cleared lysate from the ultracentrifuge was loaded onto the column, followed by a wash with 2.5 column volumes solution A. The protein was then eluted with a one column volume step with solution B (solution A plus 50 mM maltose).

Size exclusion chromatography. All protein samples were diluted in running buffer (in mM: 150 KCl, 1 or 10 CaCl₂ and/or 10 EGTA as indicated, 30 Tris, pH 8.5, and 0.5 TCEP) followed by separation on a Superdex 200 10/300 GL column (GE Healthcare) using the same running buffer at room temperature.

CaM pull-down assay. CaM agarose pull-down assay was performed as previously described (Rosenbaum et al., 2004).

Simultaneous electrophysiology and confocal imaging. Simultaneous electrophysiology-imaging experiments were performed as previously described (Klein et al., 2008). Briefly, perforated patch currents were recorded during a step depolarization (200 ms) from 0 to −60 mV using a MultiClamp 700A (Molecular Devices) interfaced to a computer controlled with Clampex 8.2. Analysis of perforated-patch currents was performed using Clampfit 8.2 (Molecular Devices). Confocal images during simultaneous electrophysiology-imaging experiments were obtained using a Radiance 2100 confocal system (Bio-Rad Laboratories) controlled with proprietary software (scanning rate of 500 lines/s). The confocal system was coupled to a Nikon TE300 inverted microscope using a Nikon 60× oil-immersion objective (numerical aperture, 1.4). PH-PLCδ1-GFP was excited using the 488 nm line of an argon laser, and emission from 515 to 530 nm was collected. Analysis of images was performed using MetaMorph 7.0 (Molecular Devices).

Reagents. All phosphoinositides were obtained from Avanti Polar Lipids. DiC8- and natural-PI(4,5)P₂ solutions were prepared as previously described (Stein et al., 2006). Polylysine (PolyK) (70–150 kDa) was dissolved as a 2 mg/ml stock, aliquoted, and frozen at -20°C . All chemicals were purchased from Sigma-Aldrich unless otherwise noted.

Data analysis. All electrophysiology data were analyzed with either Igor Pro (Wavemetrics) or GraphPad Prism 5 software. Desensitizing responses [1 min; 1 mM 2-aminoethoxydiphenyl-borate (2-APB)] were fitted by up to two exponential components. Goodness of fit was determined by visual inspection. Weighted time constants were calculated as $\tau_w = \sum(\tau_n \cdot A_n) / \sum A_n$. Comparison of the desensitization in the absence or presence of Ca^{2+} was analyzed using Student's two-tailed unpaired *t* test. Changes in fluorescence and desensitization in the simultaneous imaging and electrophysiology experiments were analyzed using Student's two-tailed paired *t* test. Changes in desensitization and current densities in the presence of CaM modulators, intracellular BAPTA, intracellular diC8PIP₂, and PIP₂ modulation of 2-APB currents on patches were analyzed using a one-way ANOVA, followed by *post hoc* Tukey's test to determine the level of significance.

Results

Ca²⁺-dependent desensitization of TRPV2 channels

We studied the mechanism of Ca²⁺-dependent desensitization of TRPV2 by measuring whole-cell currents recorded from F-11 cells transiently transfected with TRPV2 (Fig. 2). F-11 cells are derived from dorsal root ganglion (DRG) neurons (Francel et al., 1987; Caterina et al., 1999) and therefore are an excellent system to mimic TRPV2 function in native DRG neurons. TRPV2 currents were evoked by the application of 1 mM 2-APB, a nonselective TRPV2 agonist (Hu et al., 2004), at a constant holding potential of -60 mV. No 2-APB-activated currents were observed in nontransfected F-11 cells (data not shown). Acute desensitization of TRPV2 was studied with a prolonged application (60 s) of 2-APB. Application of 1 mM 2-APB induced a large current (4.1 ± 0.8 nA; $n = 14$), which, contrary to previous reports (Juvin et al., 2007; Phelps et al., 2010), showed robust desensitization when 1.8 mM Ca²⁺ was present in the extracellular solution (Fig. 2A). The rate of desensitization typically ranged in the order of seconds ($\tau_{1/2} = 18 \pm 3$ s; $n = 8$). The extent of desensitization was quantified as the residual steady-state current after 60 s relative to the peak current. In the presence of 1.8 mM Ca²⁺, TRPV2 currents were inhibited by $77 \pm 2.6\%$ ($n = 9$) (Fig. 2B). A similar, although more rapid, current inhibition ($98 \pm 0.6\%$; $n = 5$) was observed for TRPV1 in F-11 cells after a prolonged exposure to 100 μM 2-APB (Fig. 2) or 1 μM capsaicin (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) ($83 \pm 4\%$; $n = 7$) in the presence of Ca²⁺, indicating that desensitization was not agonist specific. In addition, repeated agonist applications in the presence of Ca²⁺ similarly decreased 2-APB responsiveness in F-11 cells expressing either TRPV2 or TRPV1 channels (Fig. 2A, inset; supplemental Fig. 1, available at www.jneurosci.org as supplemental material). This diminution of the maximal current amplitude after successive agonist application has been termed tachyphylaxis and is suggested to be phenomenologically different from acute desensitization. Thus, TRPV2 undergoes both acute desensitization and tachyphylaxis similar to TRPV1 channels. Importantly, little reduction of agonist-induced currents was observed when either the bath contained 1.8 mM Ca²⁺ and the pipette contained 10 mM BAPTA (supplemental Fig. 2, available at www.jneurosci.org as supplemental material) ($33 \pm 2\%$; $n = 3$) or when the bath contained 1 mM EGTA with no added Ca²⁺ and the pipette contained 10 mM BAPTA for both TRPV2 ($3 \pm 1.2\%$; $n = 5$) and TRPV1 ($3 \pm 1.6\%$; $n = 6$) (Fig. 2A). Thus, Ca²⁺ flux through the channels induced desensitization of TRPV2 channels. The strong

Figure 2. TRPV2 desensitizes in the presence of external Ca²⁺. **A**, Representative whole-cell recordings at a holding potential of -60 mV from F-11 cells transiently expressing either TRPV2 (top) or TRPV1 (bottom). TRPV2 and TRPV1 currents were evoked by a prolonged exposure (60 s) to 1 mM 2-APB or 100 μM 2-APB, respectively, either in the presence (left current traces) or absence (right current traces) of Ca²⁺. Each current represents a separate cell. A brief agonist stimulus (20 s) separated by 2 min washes with the standard bath solution containing Ca²⁺ is shown as an inset. **B**, Summary boxplot showing the amount of remaining steady-state current (60 s) relative to the peak response in the absence or presence of Ca²⁺. Boxes encompass the 25th through 75th percentile of the data, the horizontal bar represents the median, and the whiskers extend to the 10th and 90th percentile of the data. The TRPV2 and TRPV1 mean values for current remaining in the presence of Ca²⁺ are 0.2 ± 0.03 and 0.02 ± 0.006 and in the absence of Ca²⁺ are 0.97 ± 0.01 and 0.97 ± 0.02 , respectively. Data represent the mean $\pm$ SEM from at least five independent experiments. $*p < 0.0001$.

similarity between desensitization phenomena in the closely related TRPV2 and TRPV1 channels, regardless of agonist used, suggests that the mechanism of Ca²⁺-dependent desensitization might be conserved between both channels.

Ca²⁺/CaM binds to a C-terminal but not an N-terminal fragment of TRPV2

It has previously been reported that Ca²⁺/CaM may play a role in Ca²⁺-dependent desensitization of TRPV1 by binding to the N-terminal ARD (Rosenbaum et al., 2004) or by competing with ATP for a nucleotide-binding site in the ARD (Lishko et al., 2007). We wondered whether, like in TRPV1 and related TRPV channels (Warr and Kelly, 1996; Scott et al., 1997; Niemeyer et al., 2001; Tang et al., 2001; Trost et al., 2001; Zhang et al., 2001; Singh et al., 2002; Phelps et al., 2010), Ca²⁺/CaM could interact directly with the ARD of TRPV2. We tested whether the TRPV2 ARD binds Ca²⁺/CaM using a pull-down assay. As expected, the

Figure 3. TRPV2-ARD does not interact with CaM. Coomassie-stained gel of the six TRPV ARDs showing the amount of each protein that was loaded on the CaM-agarose beads (input lane), and protein bound to CaM-agarose beads in the presence of 2 mM Ca²⁺ (Ca²⁺ lane) or in the presence of 2 mM EGTA with no added Ca²⁺ (EGTA lane). Molecular weights for TRPV-GST are as follows: 76.4 kDa (TRPV1-ARD), 38.3 kDa (TRPV2-ARD), 38.6 kDa (TRPV3-ARD), 37 kDa (TRPV4-ARD), 35.4 kDa (TRPV5-ARD), and 38 kDa (TRPV6-ARD).

TRPV1-ARD GST-fusion protein bound to CaM-agarose beads in a Ca²⁺-dependent manner (Fig. 3). Consistent with previous reports (Phelps et al., 2010), at least some binding was also observed for TRPV3-ARD, TRPV4-ARD, TRPV5-ARD, and TRPV6-ARD, and this binding required the presence of Ca²⁺. However, for TRPV2-ARD, binding to CaM-agarose was not observed, either in the presence or absence of Ca²⁺ (Fig. 3). We conclude that Ca²⁺/CaM does not interact with the ARD of TRPV2 and thus that a role for a Ca²⁺/CaM interaction with the ARD of TRPV2 in Ca²⁺-dependent desensitization seems unlikely.

It has been proposed that binding of Ca²⁺/CaM to the distal C-terminal region of TRPV1 plays a role in TRPV1 Ca²⁺-dependent desensitization (Numazaki et al., 2003). We tested whether a similar binding site might be present in TRPV2 by studying a C-terminal fragment of TRPV2, corresponding to amino acids 684–753, fused to maltose binding protein as an affinity purification tag (TRPV2-C-MBP). We coexpressed this construct in bacteria with a second plasmid containing the cDNA for CaM. We found that, on affinity purification of the TRPV2-C-MBP fusion protein, CaM was purified as well (Fig. 4A).

To test whether the TRPV2 C-terminal fusion protein was monodispersed or aggregated, we ran an affinity-purified sample on a size exclusion column. As shown in Figure 4B, the TRPV2-C-GFP fusion construct eluted as two peaks, one a proteolytic fragment corresponding to green fluorescent protein (GFP) alone and a second corresponding to the fusion protein (Fig. 4B, black trace). The monodispersion of the fusion protein indicates that its copurification with CaM was not attributable to nonspecific interactions with aggregated protein.

The binding of Ca²⁺/CaM could be observed in size exclusion chromatography experiments. When added to TRPV2-C-GFP, Ca²⁺/CaM produced a shift in the fusion peak toward lower elution volumes (Fig. 4B, blue trace). Indeed, light scattering size exclusion chromatography showed a 1:1 stoichiometry for the TRPV2-C:CaM complex (data not shown). Coexpression of TRPV2-C-GFP and CaM in the same bacteria not only produced

TRPV2-C-GFP:CaM complex but also increased the relative fraction of monodispersed fusion protein (Fig. 4B, red trace vs blue trace). The complex dissociated on the addition of 10 mM EDTA (Fig. 4C). Thus, the TRPV2 C-terminal fusion protein bound CaM in a Ca²⁺-dependent manner.

The CaM-binding domain of myosin light chain kinase (MLCK), a 17 aa peptide, binds CaM with high affinity ($K_D = 6$ μ M) (Török et al., 1998). To determine whether Ca²⁺/CaM bind specifically to the TRPV2 C-terminal fragment, we tested whether the MLCK peptide could compete with TRPV2-C-MBP for binding to CaM. We found that the addition of 1 μ M of the MLCK peptide to the TRPV2-C-MBP protein coexpressed with CaM produced a shift in the TRPV2-C-MBP fusion peak (Fig. 4D, green trace) similar to that produced by addition of EDTA (Fig. 4C, black trace). These data suggest that the binding of CaM to the C-terminal fragment of TRPV2 is specific and reversible.

To identify the region(s) in the C terminus of TRPV2 involved in CaM binding, two TRPV2-C-GFP fusion constructs were generated: a proximal construct containing the first 39 residues (TRPV2#645–684-C-GFP) and a distal construct containing the last 49 residues of the TRPV2 C terminus (TRPV2#704–753-C-GFP). Both constructs were soluble and monodispersed as determined by size exclusion chromatography (Fig. 4E, F, black trace). However, only TRPV2#645–684-C-GFP bound Ca²⁺/CaM, as revealed by the CaM-dependent shift in the elution volume from a size exclusion column (Fig. 4E, red trace) indicating that this 39 residue fragment is sufficient for binding of CaM to the C terminus of TRPV2 *in vitro*.

Ca²⁺/CaM is not a major player in TRPV2 desensitization

Based on our biochemistry results (Fig. 4), we hypothesized that CaM binds to the TRPV2 proximal C-terminal region and acts as a Ca²⁺ sensor, modulating TRPV2 channel activity in response to increases in intracellular Ca²⁺ concentration. We assessed the physiological importance of CaM binding to the TRPV2 C-terminal region by recording from intact TRPV2 channels using the patch-clamp technique. Previously, we showed that the MLCK peptide could remove CaM from the TRPV2 C-terminal region (Fig. 4D). We therefore applied the MLCK peptide through the patch pipette in the whole-cell configuration. Application of 1 mM 2-APB in the presence of 1.8 mM Ca²⁺ to cells dialyzed with 1 μ M MLCK caused TRPV2 currents to desensitize by $79 \pm 4\%$ (I_{ss}/I_{peak} ; $n = 3$) similar to TRPV2 currents without peptide (I_{ss}/I_{peak} , $77 \pm 2.6\%$; $n = 9$) (Fig. 5). Thus, intracellular infusion of 1 μ M MLCK did not significantly affect TRPV2 desensitization.

Previous studies on TRPV1 channels have suggested that CaM is already associated with the channel even in the absence of Ca²⁺ (Numazaki et al., 2003; Rosenbaum et al., 2004). Although our biochemistry results suggest that 1 μ M MLCK peptide is sufficient to effectively compete with TRPV2 for binding to CaM, the negative results with MLCK peptide might be explained by the inability of the MLCK peptide to compete with endogenous CaM preassociated with TRPV2 channels. We, therefore, coexpressed TRPV2 channels with a nonfunctional CaM (CaM₁₂₃₄), a mutant in which all four Ca²⁺ binding sites have been crippled. If there is a Ca²⁺-free association, overexpressed CaM₁₂₃₄ ought to compete with wild-type CaM for association with the channels, but it should not be able to transduce the changes in Ca²⁺ concentration into changes in channel function. Cotransfection of TRPV2 with CaM₁₂₃₄ also failed to prevent TRPV2 desensitization (I_{ss}/I_{peak} , $74 \pm 5\%$; $n = 6$) (Fig. 5). No significant differences were observed in current densities between

cells coexpressing TRPV2 and CaM_{1234} (618 ± 162 pA/pF; $n = 6$) and cells expressing TRPV2 dialyzed with the MLCK peptide (611 ± 307 pA/pF; $n = 3$) when compared with TRPV2 alone (419 ± 68 pA/pF; $n = 7$) ruling out differences in the amount of Ca^{2+} entry. Overall, these results suggest that CaM binding to the proximal C-terminal region of TRPV2 may not be functionally coupled to desensitization of TRPV2. Alternatively, CaM might not bind to the C terminus of intact TRPV2 channels, as it does for the C-terminal fragments *in vitro*.

TRPV2 Ca^{2+} -dependent desensitization is mediated by PIP_2 depletion

Recent evidence indicates that a growing number of mammalian TRP channels are functionally regulated by phosphoinositide 4,5-bisphosphate (PIP_2) (Runnels et al., 2002; Lee et al., 2005; Rohács et al., 2005; Zhang et al., 2005; Nilius et al., 2006; Stein et al., 2006; Karashima et al., 2008; Klein et al., 2008; Thyagarajan et al., 2009). It has been proposed that Ca^{2+} entry through TRPV1 channels can activate a calcium-sensitive phospholipase C (PLC) to reduce the concentration of PIP_2 in the plasma membrane (Lukacs et al., 2007). We examined whether Ca^{2+} entry through TRPV2 channels would induce degradation of PIP_2 in the plasma membrane. As a marker for PIP_2 , we used a fluorescent probe, GFP fused to the PLC δ 1-PH domain (GFP-PLC δ 1-PH). We transiently transfected F-11 cells with either TRPV2 and GFP-PLC δ 1-PH or GFP-PLC δ 1-PH alone. Confocal imaging shows that GFP-PLC δ 1-PH was localized to the plasma membrane (Fig. 6) as expected if it bound to the relatively high concentration of PIP_2 in the membrane (Várnai and Balla, 1998). Application of 1 mM 2-APB in the presence of Ca^{2+} induced a robust translocation of the GFP-PLC δ 1-PH from the plasma membrane to the cytosol in F-11 cells cotransfected with TRPV2 (Fig. 6A; supplemental Movie 1, available at www.jneurosci.org as supplemental material). In contrast, translocation did not occur in F-11 cells not transfected with TRPV2 (Fig. 6B) or in the absence of extracellular Ca^{2+} (supplemental Movie 1, available at www.jneurosci.org as supplemental material). Thus, Ca^{2+} entry through TRPV2 resulted in a decrease in PIP_2 levels in the plasma membrane possibly via activation of a calcium-sensitive PLC.

If the reduction in plasma membrane PIP_2 contributes directly to Ca^{2+} -dependent desensitization of TRPV2, then changes in PIP_2 levels should precede or coincide with desensitization. We tested this hypothesis using simultaneous confocal imaging and whole-cell electrophysiology measurements. We co-

Figure 4. CaM forms a Ca^{2+} -dependent complex with a TRPV2 C-terminal region. **A**, Coomassie-stained gel of TRPV2-C-MBP: CaM complex on affinity purification of the TRPV2-C-MBP fusion protein from bacteria expressing both CaM and TRPV2-C-MBP. **B–D**, When coexpressed with CaM (**B–D**, red trace) or mixed together with exogenous CaM (**B**, blue trace) in the presence of 10 mM Ca^{2+} , the TRPV2-C-GFP fusion construct (**B**, black trace) elute as a higher molecular weight complex from a size exclusion chromatography column. This complex is disrupted by replacement of Ca^{2+} with 10 mM EDTA (**C**, black trace). **D**, TRPV2-MBP fusion construct coexpressed with CaM , purified as shown in **A**, was injected on a size exclusion chromatography column in the absence (red trace) or presence of 1 μM MLCK peptide (green trace), all in the presence of 10 mM Ca^{2+} . **E**, **F**, When coexpressed with CaM , a proximal C-terminal-C-GFP fusion construct (TRPV2#645–684-C-GFP) eluted as a higher molecular weight complex from a size exclusion column (**E**, red trace), but a distal C-terminal fusion construct (TRPV2#704–753-C-GFP) did not (**F**, red trace).

expressed TRPV2 and GFP-PLC δ 1-PH in tsA201 cells (Fig. 7A) and recorded the localization of the GFP-PLC δ 1-PH probe at the same time as we measured TRPV2 current (Fig. 7A, B; supplemental Movie 1, available at www.jneurosci.org as supplemental material). We first activated the TRPV2 channels with 2-APB in a Ca^{2+} -free bath (Fig. 7B, white bar). Neither the translocation of the GFP-PLC δ 1-PH PIP_2 probe (Fig. 7B, red trace; C) (cytosolic F/F_0 , 0.9 ± 0.02) nor desensitization of the TRPV2 currents (Fig. 7B, black trace; C) (I_{ss}/I_{peak} , $97 \pm 1\%$) occurred in the absence of extracellular Ca^{2+} . Next, we added 1.8 mM free Ca^{2+} in the presence of 2-APB (Fig. 7B, gray bar; supplemental Movie 1, available at www.jneurosci.org as supplemental material). Addition of Ca^{2+} to the bath caused a rapid increase in the cytosolic GFP-

Figure 5. Inhibiting CaM does not prevent TRPV2 Ca²⁺-dependent desensitization. **A**, Representative whole-cell TRPV2 currents in control, cells dialyzed with the MLCK peptide, or cells cotransfected with CaM₁₂₃₄. MLCK peptide, at a concentration of 1 μ M, was dialyzed through the patch pipette. For MLCK peptide experiments, recordings were performed 5 min after the formation of whole-cell configuration. **B**, Summary boxplot showing the amount of remaining steady-state current relative to the peak response. The TRPV2 mean value for current remaining in cells cotransfected with CaM₁₂₃₄ is 0.3 ± 0.05 and 0.21 ± 0.04 in cells dialyzed with 1 μ M MLCK peptide. TRPV2 mean values for current remaining in the presence and absence of Ca²⁺ reported on Figure 2 are shown for comparison. Data represent the mean $\pm$ SEM from at least three independent experiments. * $p < 0.0001$.

PLC δ 1-PH fluorescence (Fig. 7B, red trace; C) (cytosolic F/F_0 , 2 ± 0.1) that coincided with TRPV2 channel desensitization (Fig. 7B, black trace; C) (I_{ss}/I_{peak} , $20 \pm 3\%$). The coincidence between PIP₂ degradation and TRPV2 desensitization indicates that the two processes, PIP₂ hydrolysis and TRPV2 desensitization, are coupled to Ca²⁺ flux through the channels. These data support the hypothesis that hydrolysis of PIP₂ by PLC underlies Ca²⁺-dependent desensitization of TRPV2 channels.

PIP₂ inhibits desensitization in cells and potentiates TRPV2 in excised patches

Our data suggest that hydrolysis of PIP₂ may underlie desensitization of TRPV2 channels. We propose that the mechanism involves a direct interaction of PIP₂ and TRPV2, such that TRPV2 activation is favored when PIP₂ is bound, and deactivation (or desensitization) is favored when PIP₂ is degraded.

To test our hypothesis, we performed whole-cell patch-clamp experiments in F-11 cells expressing TRPV2 in which diC8-PIP₂

(100 μ M) was included in the whole-cell patch pipette (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). If TRPV2 desensitization is favored when PIP₂ is degraded, replenishing the membrane with diC8-PIP₂ dialyzed into the cell via the patch pipette ought to decrease TRPV2 desensitization. Indeed, inclusion of diC8-PIP₂ in the whole-cell patch pipette caused a significant reduction in TRPV2 desensitization (I_{ss}/I_{peak} , $36 \pm 5\%$; $n = 3$) (supplemental Fig. 3B, available at www.jneurosci.org as supplemental material) when compared with control conditions (I_{ss}/I_{peak} , $77 \pm 2.6\%$; $n = 9$). The incomplete inhibition of TRPV2 desensitization by inclusion of diC8-PIP₂ in the intracellular pipette solution suggest either an inefficient delivery of diC8-PIP₂ to the plasma membrane compared with the efficiency of hydrolysis by PLC or the involvement of additional mechanisms in TRPV2 Ca²⁺-dependent desensitization.

We next asked whether PIP₂ can act directly on the channels or, alternatively, requires an intact cellular environment. Our strategy involved application of the PIP₂-sequestering agent PolyK to inside-out excised patches followed by application of either diC8-PIP₂ or natural PIP₂. We found that TRPV2 currents in inside-out patches underwent an activity-dependent rundown during 2-APB application (data not shown). To minimize rundown during our experiments, we used low concentrations (≤ 100 μ M) of 2-APB, in which the currents remained stable. To examine the direct effect of PIP₂ on TRPV2 channels, we applied PolyK, a polycation that sequesters PIP₂ (Toner et al., 1988; Gabev et al., 1989; Ben-Tal et al., 1996), to the intracellular surface of inside-out patches (Fig. 8A). Application of PolyK (30 μ g/ml) to the inner leaflet of the membrane inhibited 2-APB-induced currents, suggesting that negatively charged lipids are required for 2-APB-mediated activation of TRPV2 channels. The mean values for current remaining ($I/I_{initial}$) after PolyK treatment was 0.2 ± 0.03 (Fig. 8). Although the currents did not recover on removal of PolyK from the bath, they were rescued by the addition of either natural PIP₂ ($I/I_{initial}$, 0.8 ± 0.2 ; $n = 3$) or diC8-PIP₂ ($I/I_{initial}$, 2 ± 0.2 ; $n = 7$), respectively (Fig. 8) (see Materials and Methods). We conclude that PIP₂ can substitute for the activating molecule sequestered by PolyK. In sum, our data indicate that TRPV2 channels is activated by the direct binding of PIP₂, and that desensitization arises from the hydrolysis of PIP₂ that results from Ca²⁺ entry through the channels.

Discussion

Here, we report that TRPV2 channels undergo Ca²⁺-dependent desensitization, similar to the closely related TRPV1 channel. Potential molecular mechanisms for Ca²⁺-dependent desensitization of TRPV2 were studied using electrophysiology as well as fluorescence-based methods. We found that, although CaM binds to a proximal C-terminal fragment of TRPV2 in our *in vitro* assay, CaM does not play a major role in TRPV2 Ca²⁺-dependent desensitization. In contrast, decreases in membrane PIP₂ levels on channel activation underlie a major component of the Ca²⁺-dependent desensitization observed on TRPV2 channels. Together, these results indicate that changes in membrane PIP₂ levels are responsible for much, if not all, the TRPV2 Ca²⁺-dependent desensitization and raise the question whether this mechanism is conserved among other TRP channels with similar Ca²⁺-dependent desensitization.

Historically, two types of desensitization have been described for TRPV1 channels: acute desensitization and tachyphylaxis. Acute desensitization refers to a decrease in the current amplitude seen in response to a long, continuous application of the

ligand, whereas tachyphylaxis refers to the reduction in evoked current when very brief applications of the ligand are made several minutes apart (Koplas et al., 1997). With such protocols, Ca²⁺-dependent desensitization of TRPV1 has been found to depend on a number of intracellular factors, including ATP (Koplas et al., 1997), calcineurin (Docherty et al., 1996), PKA (Bhave et al., 2002; Mohapatra and Nau, 2003), PKC (Mandadi et al., 2004), PIP₂ (Liu et al., 2005; Rohács et al., 2005; Lishko et al., 2007; Yao and Qin, 2009; Phelps et al., 2010), and CaM (Numazaki et al., 2003; Rosenbaum et al., 2004). However, the brief stimuli used in tachyphylaxis present limitations on the mechanistic interpretation of the data since they convolve multiple factors including channel activation, acute desensitization, and recovery from desensitization. In this study, we used prolonged applications of the ligands at a relatively high concentration to ensure maximal or near-maximal desensitization of the channels. With this protocol, we found that the desensitization of TRPV2 channels (Fig. 2) is typical of its subfamily in several aspects: (1) it depends on extracellular Ca²⁺, (2) the desensitization rate is on the order of seconds, and (3) the channel enters into a refractory period where channel activation has significantly decreased (Fig. 2, inset). Thus, although TRPV2 channels might mediate a completely different range of physiological and sensory responses than other TRPV channels (Caterina et al., 1999; Muraki et al., 2003; Stokes et al., 2004; Link et al., 2010), they share a common structural architecture (Fig. 1) (Caterina et al., 1999) and a common Ca²⁺-dependent desensitization with other TRPV channels (Fig. 2).

What is the molecular mechanism responsible for Ca²⁺-dependent desensitization in TRPV2? The Ca²⁺ dependence of CaM binding to the proximal TRPV2 C terminus (Fig. 4) presents an attractive model where Ca²⁺ entering through the channels can generate TRPV2-CaM/Ca²⁺ complexes, leading to TRPV2 channel desensitization. However, our negative results with the CaM-scavenging peptide (MLCK peptide) and the mutant CaM (CaM₁₂₃₄) (Fig. 5) suggest that CaM/Ca²⁺ binding to the proximal TRPV2 C terminus is not coupled to TRPV2 desensitization. It is also unlikely that CaM/Ca²⁺ binding to the TRPV2 N terminus, another potential cytosolic domain for CaM binding, is involved in TRPV2 desensitization since recent studies (Lishko et al., 2007; Phelps et al., 2010) and our biochemistry results (Fig. 3) indicate that the N terminus of TRPV2 does not interact with CaM. Although we cannot rule out an indirect role for CaM in Ca²⁺-dependent desensitization, a direct interaction with the channels does not appear to regulate desensitization of TRPV2.

Changes in the plasma membrane PIP₂ concentration have been implicated in the regulation of many membrane proteins, in particular, ion channels (for review, see Nilius et al., 2008; Rohács, 2009). PIP₂ depletion, however, occurs in many cases, through activation of a second messenger signaling pathway mainly involving G-protein-coupled receptors (Suh et al., 2004;

Figure 6. Ca²⁺ entry through TRPV2 channels induces PIP₂ depletion. F-11 cells were transfected with either TRPV2 and a GFP fused to the PLCδ1-PH domain (GFP-PLCδ1-PH) that is targeted to the plasma membrane through binding to PIP₂ (**A**) or with GFP-PLCδ1-PH only (**B**). Shown are confocal images of GFP-PLCδ1-PH fluorescence from representative cells before and after addition of 1 mM 2-APB in the constant presence of Ca²⁺. PIP₂ depletion was assessed by the translocation of the GFP-PLCδ1-PH from the plasma membrane to the cytosol. Scale bar: 5 μm (applies to all images). The right panel shows summary boxplot of normalized fluorescence intensity of GFP in the cytosol for each condition. Data represent the mean ± SEM from at least three independent experiments. **p* < 0.0001.

Horowitz et al., 2005). In particular, TRPV2 channel trafficking to the plasma membrane has been shown to be regulated by insulin-like growth factor-I (Kanzaki et al., 1999). Here, we have shown that TRPV2 is also sensitive to changes in membrane PIP₂ (Figs. 7, 8). The rapid depletion of PIP₂ on TRPV2 activation, however, suggests that Ca²⁺ entry through the channel is sufficient to trigger PIP₂ depletion without the need for an auxiliary surface receptor. In addition, we have shown that the effect of PIP₂ in TRPV2 channels is a direct modulation rather than through auxiliary mechanisms (Fig. 8; supplemental Fig. 3, available at www.jneurosci.org as supplemental material) such as trafficking of the channel in and out of the plasma membrane. Thus, TRPV2 acts as both the auxiliary receptor and PIP₂ target. In this way, the entry of Ca²⁺ through open channels could feedback on the channel within seconds to cause the channel to close.

TRPV2 recovery from desensitization would presumably require resynthesis of PIP₂. It has been suggested that resynthesis of membrane PIP₂ determines recovery of TRPV1 channels from desensitization (Liu et al., 2005). The production of phospholipids by lipid kinases generally takes minutes and require a significant amount of intracellular ATP (≥1 mM) (Várnai and Balla, 1998; Suh and Hille, 2002), involving a series of steps including phosphorylating phosphatidylinositol lipid on multiple positions. The proposed slow PIP₂-dependent recovery is consistent with the observed failure of the channel to recover from desensitization during our “tachyphylaxis” protocol (Fig. 2, insets) as has been suggested for TRPV1 (Liu et al., 2005).

Is PIP₂ depletion solely responsible for TRPV2 desensitization? Although we cannot exclude a role for other molecular mechanisms in shaping various aspects of TRPV2 desensitization, our data suggest that PIP₂ can directly regulate the channel and that PIP₂ depletion induces a significant desensi-

Figure 7. Depletion of PIP₂ is coincident with TRPV2 desensitization. **A**, **B**, **A** representative imaging and electrophysiology experiment for Ca^{2+} -induced PIP₂ depletion in TRPV2 channels. **A**, GFP-PLC δ 1-PH signal in a tsA cell transfected with TRPV2 and GFP-PLC δ 1-PH probe. Shown are confocal images for a time point in the current and fluorescence traces shown in **B**. Scale bar: 5 μ m (applies to all images). **B**, Traces represent the mean fluorescent data (top) from two different cytosolic regions of interest and the current (bottom) from the cell depicted in **A** with an arrow. Currents (perforated-patch configuration; holding potential, -60 mV) were elicited by 1 mM 2-APB in a Ca^{2+} -free solution and changed to a Ca^{2+} -containing solution (1.8 mM Ca^{2+}) in the continuous presence of the channel agonist to elicit desensitization. An average perfusion exchange delay of 47 s between Ca^{2+} -free and Ca^{2+} -containing solutions was observed for all the experiments and is shown in this figure (dotted line) for illustration purposes. **C**, Summary boxplot showing normalized fluorescence intensity of GFP in the cytosol (left) and amount of current inhibition (right) using the same conditions as in **B**. Values for fluorescence and current were taken at the same time point. The mean value for fluorescent intensity and current remaining in the absence of Ca^{2+} (open boxes) is 0.9 ± 0.02 and 0.97 ± 0.01 and in the presence of Ca^{2+} is 2.0 ± 0.1 and 0.2 ± 0.03 , respectively. Data represent the mean $\pm$ SEM from four independent experiments. * $p < 0.0002$.

tization of the channel. Consistent with this hypothesis, a recent study has shown that TRPV2 does not bind several of the ligands (e.g., ATP, calmodulin) proposed to mediate Ca^{2+} -dependent desensitization in TRPV1 (Numazaki et al., 2003; Lishko et al., 2007; Phelps et al., 2010). Thus, for TRPV2 channels, PIP₂ depletion is likely to be the major molecular player in TRPV2 desensitization.

The Ca^{2+} dependence and stereotyped form of desensitization observed in many TRP channels suggests that some regulatory pathways may be conserved. In particular, TRPV1 and TRPV2 are closely related, with 49% (Caterina et al., 1999) amino acid identity, and show remarkably similar Ca^{2+} -dependent desensitization (Fig. 2). Other common properties of TRPV1 and

Figure 8. Natural and diC8-PIP₂ rescue inhibition of TRPV2 by PolyK. **A**, Representative time courses of TRPV2 activation in inside-out excised patches from F-11 cells. A voltage protocol (inset) was used to drive current through the channel and 100 μ M 2-APB was applied to the patch to elicit TRPV2 current. PolyK (30 μ g/ml) or diC8-PIP₂ (10 μ M) were applied as indicated. **B**, Summary boxplot of the fractional inhibition by PolyK and the restoration by PIP₂. The mean values for current remaining ($I/I_{initial}$) after PolyK treatment is 0.2 ± 0.03 and 0.3 ± 0.04 after washing the PolyK from the bath. Data represent the mean $\pm$ SEM from at least three independent experiments. * $p < 0.0001$, significantly different from PolyK wash.

TRPV2 include the following: activation by a common ligand (2-APB), voltage dependence of activation, activation by heat (albeit with different thresholds), and functional heteromultimerization between TRPV1 and TRPV2 channels (Hellwig et al., 2005; Liapi and Wood, 2005). It is therefore attractive to consider that the molecular mechanism of Ca^{2+} -dependent desensitization in TRPV1 and TRPV2 is conserved. Although ATP and CaM have been shown to bind to the ankyrin repeat domain in the N terminus of TRPV1 and proposed to play important roles in Ca^{2+} -dependent desensitization, these factors do not bind to the ankyrin repeat domain of TRPV2 (Fig. 3) (Lishko et al., 2007; Phelps et al., 2010). As well, we found that a potential CaM binding site in the TRPV2 C-terminal region is not coupled to TRPV2 desensitization. The simplest interpretation of our data is that ATP and CaM play at most minor roles in Ca^{2+} -dependent desensitization of TRPV2 and TRPV1. Together with previous reports, our work indicates that Ca^{2+} -dependent degradation of PIP₂ represents the major mechanism involved in Ca^{2+} -dependent desensitization of TRPV2 and TRPV1 and raises the question of whether this mechanism is conserved in other TRP channels.

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