Involvement of Phosphatidylinositol 4,5-Bisphosphate in the Desensitization of Canonical Transient Receptor Potential 5

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The classic transient receptor potential channel (TRPC) is a candidate for Ca2+-permeable cation channel in mammalian cells. TRPC5 is desensitized rapidly after activation by G protein-coupled receptor. Here we investigate the mechanisms of desensitization of TRPC5 using patch-clamp recording. TRPC5 was initially activated by muscarinic stimulation using 50 μM carbachol (CCh) and decayed rapidly in the presence of CCh (desensitization). Intracellularly-applied phosphatidylinositol 4,5-bisphosphate (PIP2) slowed the rate of desensitization. In contrast, several other phosphoinositides, including PI(3,4)P2, PI(3,5)P2, PI(3,4,5)P3 and PI(4)P, had no effect on the desensitization of the TRPC5 current. This indicates that PIP2 attenuates the desensitization of the TRPC5 current in a highly selective manner. Neither wortmannin, an inhibitor of phosphatidylinositol 4-kinase, or poly-L-lysine (PLL), a scavenger of PIP2, had any effect on desensitization of the TRPC5 current. PIP2 breakdown appears to be a required step in the desensitization of TRPC5 current, but PIP2 depletion alone was insufficient for channel desensitization. TRPC5 was inhibited by cytochalasin D treatment. In mouse ileal myocytes, the desensitization of CCh-activated inward current (I_{CCh}) also slowed in the presence of PIP2 in recording pipettes. These results indicate that PIP2 is involved in the desensitization of TRPC5 currents.

Key words  canonical transient receptor potential 5; muscarinic stimulation; phosphatidylinositol 4,5-bisphosphate

MATERIALS AND METHODS

Cell Culture and Transient Transfection  Human embryonic kidney (HEK)293 cells (ATCC, Manassas, VA, U.S.A.) were maintained according to the recommendations of the supplier. For transient transfection, cells were seeded in 6-well plates. The following day, 1 μg/well pcDNA plasmid vectors containing the cDNA for TRPC5-GFP (given kindly by Dr. Shuji Kaneko) and muscarinic receptor 3 were transfected into cells using the transfection reagent FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer’s protocol. After 24—48 h, cells were trypsinized and used for whole-cell recording.

Dissociation of Single Cells from Mouse Ileum  Ileal myocytes were enzymatically isolated from the ileal region of Institute for Cancer Research (ICR) mice. Mice of either sex weighing 20—30 g were anaesthetized with carbon dioxide and sacrificed by cervical dislocation. The ileal part of the intestine was cut out. The mucous layer was dissected from the smooth muscle layer with fine scissors and cut into small segments (ca. 2—3 mm). Segments were then digested for 25—30 min at 37 °C in Ca2+-free Tyrode solution containing 0.1% collagenase (Worthington type 2), 0.1% dithiothreitol, 0.1% trypsin inhibitor, and 0.2% bovine serum albumin. Single myocytes were dispersed by gentle agitation of the digested segments with a wide-bored glass pipette and kept at 4 °C until use. All experiments were carried out at room temperature within 10 h of harvesting cells.

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Whole-Cell Patch-Clamp Recording Isolated cells were transferred to a small chamber on the stage of an inverted microscope (IX70, Olympus, Japan). Cells were continually perfused with normal Tyrode solution (2—3 ml/min). A glass microelectrode with a resistance of 3—5 MΩ was used to make a gigaohm seal. The conventional whole-cell patch-clamp technique was adapted to hold the membrane potential at ~60 mV using an Axopatch 200B patch-clamp amplifier (Axon Instrument, U.S.A.). For data acquisition and the application of command pulses, pCLAMP software (v.9.2) and Digidata 1200 (Axon Instruments) were used. Data were analyzed at 5 kHz and displayed on a computer monitor; they were analyzed using pCLAMP and Origin software (Microcal origin v.7.0, U.S.A.).

Solutions and Drugs Tyrode solution contained 135 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 10 mM HEPES (N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulphonic acid]). The pH was adjusted to 7.4 using NaOH. Cs-rich external solution was made by replacing NaCl and KCl with equimolar CsCl. The pipette solution contained 140 mM CsCl, 10 mM HEPES, 0.2 mM Tris–GTP (Tris–guanosine 5’–triphosphate), 0.5 mM ethylene glycol-bis-(2-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), and 3 mM Mg–ATP (Mg–adenosine 5’–triphosphate). The pH was adjusted to 7.3 with CsOH. Stock solutions of the DMSO-soluble drugs cytochalasin D (5 mM) and wortmannin (100 mM) were made. DiC8-phosphoinositides (Echelon Research Laboratories, Salt Lake City, UT, U.S.A.) were dissolved in deionised and deoxygenated water (0.5 mM) or directly in the pipette solution (if applied at concentrations higher than 20 μM) by a 30-min sonication. All other drugs were obtained from Sigma (U.S.A.).

Statistics All data are expressed as means±S.E.M. Statistical significance was determined using Student’s unpaired t-tests. p values <0.05 were considered statistically significant. The number of cell recordings in each experiment is represented by n.

RESULTS

Effects of PIP₂ on Desensitization of the TRPC5 Current Whole-cell currents were recorded using patch-clamp techniques. Initially these currents were recorded in the presence of normal Tyrode solution. Then, the external solution was changed from normal Tyrode to 140 mM [Cs⁺]o, resulting in a slight increase in basal currents, due to the constitutive activity of TRPC5. At times, currents up to 1 nA were activated without stimulation by CCh. Therefore, we waited at least 2 min before the application of CCh. Whole-cell currents also were recorded in a solution containing 140 mM extracellular Cs⁺ concentration ([Cs⁺]o) and [Cs⁺], as a control to determine the I–V relationship of TRPC5 activated by CCh. To examine I–V relationships, we applied a ramp pulse from +100 mV to −100 mV for 500 ms. When 50 μM CCh was added at a holding potential of −60 mV, an inward current was activated in HEK293 cell expressing TRPC5 (Fig. 1Aa). The I–V relationship, obtained by subtracting the current observed in the absence of CCh from the current obtained in its presence, showed a typical doubly rectifying shape (Fig. 1Ab). TRPC5 currents were not activated by CCh in mock transfected cells (Fig. 1B). We used only results obtained from cells producing the typical I–V relationship of TRPC5. The TRPC5 current that was activated by stimulating muscarinic receptors decayed spontaneously to basal levels, even during CCh application (Fig. 1Aa). This process is referred to as desensitization. Previous studies assumed that the depletion of PIP₂ by the hydrolysis of PIP₂ through PLCβ induces the activation of TRPC5. We first examined the role of the PLC substrate, PIP₂, on the TRPC5 current. To study the effects of PIP₂ on the agonist-induced TRPC5 current, we included 20 μM dioctanoyl analog of PIP₂ (diC8-PIP₂), a more water-soluble short form of PIP₂, in the recording pipettes. Under these conditions, CCh induced TRPC5 cur-
rents, contrary to what was expected. However, the desensitization of the TRPC5 current slowed in response to CCh (Fig. 1Ca). During desensitization, the \( I-V \) relationship showed a typical doubly rectifying shape (Fig. 1Cb). In TRPC5-expressing cells, the inward current spontaneously declined to 1.6\( \pm \)0.3\% of the initial peak amplitude found 2 min after CCh application (\( n=20 \)). In TRPC5-expressing cells included PIP\(_2\) in recording pipettes, however, the inward current remained at 57\( \pm \)10\% of the initial peak amplitude 2 min after CCh application. There was no significant change in the activation process (\( n=10 \)) (Fig. 1D). The decay rates were significantly different. Montell and colleagues\(^{12}\) have recently demonstrated the direct binding of several phosphoinositides (PIs) to other members of the TRP superfamily, including TRPC1, 5, 6 and 7. Therefore, we examined the effects of other phosphoinositides (PIs) on TRPC5 currents. We investigated the effect of PI(3,4,5)P\(_3\), PI(3,4)P\(_2\), PI(3,5)P\(_2\), and PI(4)P. Taking into account differences in the binding affinities,\(^{12}\) PIs were applied (similarly to PIP\(_2\)) at the following concentrations: PI(3,4,5)P\(_3\) (\( n=7 \)) at 20 \( \mu \)M, PI(3,4)P\(_2\) (\( n=3 \)) and PI(3,5)P\(_2\) (\( n=4 \)) at 100 \( \mu \)M, and PI(4)P (\( n=3 \)) at 200 \( \mu \)M. No attenuation of desensitization was seen (Fig. 2), highlighting the specificity of the attenuation of PIP\(_2\) on the desensitization of the TRPC5 current. This result is not simply a negative charge effect of PIP\(_2\).

**PIP\(_2\) Depletion Is Not Sufficient for Full TRPC5 Desensitization**

Wortmannin, an inhibitor of phosphatidylinositol 4-kinase, retards the replenishment of PIP\(_2\), which leads to depletion of the intracellular PIP\(_2\) pool.\(^{22}\) In the whole-cell mode, 50 \( \mu \)M wortmannin in the pipette solution did not have any effect on desensitization of the TRPC5 current (Fig. 3Aa) or on the typical doubly rectifying \( I-V \) shape (Fig. 3Ab). Poly-L-lysine (PLL) is a positively charged macromolecule that acts as a scavenger of PIP\(_2\).\(^{23,24}\) However, 3 \( \mu \)g/ml PLL in the pipette solution did not cause any significant change in desensitization of the TRPC5 current (Fig. 3Ba) or on the typical doubly rectifying \( I-V \) shape (Fig. 3Bb). In TRPC5-expressing cells, the desensitization of the TRPC5 current, by the addition of wortmannin and PLL to the
recording pipettes, had no significant effects \((n=5)\) (Fig. 3C). Zhu et al.\(^{17}\) reported that EGTA, depletion of Mg–ATP, or PKC inhibitor slows the desensitization. Therefore, we examined the effect of wortmannin and PLL on desensitization of the TRPC5 current in the presence of EGTA or PKC inhibitor. In this paper, we performed all experiments with internal solution containing 0.5 mM EGTA. In an internal solution containing 2 mM EGTA, the desensitization of TRPC5 current was attenuated.\(^{17}\) When we applied wortmannin or PLL in 2 mM EGTA pipette solution, there was no effect of wortmannin or PLL on desensitization of TRPC5 current (Fig. 4A). PKC inhibitor slowed the desensitization of TRPC5 current.\(^{17}\) When we applied wortmannin or PLL in pipette solution and PKC inhibitor GF109203X 100 nM in bath solution, CCh (50 μM) induced an inward current, which slowed desensitization during CCh treatment.

The Role of the Actin Cytoskeleton on TRPC5 Current

PIP2 interacts with cytoskeletal proteins and anchors various signalling molecules to the plasma membrane.\(^{26,27}\) Proteins involved in cytoskeletal remodeling are important targets of PIP2 signalling. Therefore, we investigated whether the association of PIP2 with the actin cytoskeleton has a role in PIP2 regulation of desensitization of the TRPC5 current. TRPC5-expressing cells were treated with 0.5 μM cytochalasin D, a potent cell permeable fungal toxin, which inhibits actin polymerization and thus disrupts actin microfilaments. Cytochalasin D completely inhibited the activation of the TRPC5 current (Fig. 4), such that it was impossible to investigate whether the association of PIP2 with the actin cytoskeleton has a role in regulating desensitization of this current. Cytochalasin D also inhibited the desensitization of TRPC5 current when it was added after the activation of TRPC5 current (Fig. 5B). These results suggest that the cytoskeleton is involved in both activation and desensitization of TRPC5 current.

**PIP2 Attenuates Desensitization of the CCh-Activated Current \(I_{\text{CCh}}\) in Mouse Ileal Myocyte**

Since \(I_{\text{CCh}}\) appears to be largely mediated by TRPC5,\(^ {3,21}\) it was important to determine whether desensitization of \(I_{\text{CCh}}\) depends on PIP2. CsCl-rich solutions were used in both the pipette and bath to record \(I_{\text{CCh}}\). Under a voltage clamp, at a holding potential of \(-60\) mV, 50 μM CCh induced \(I_{\text{CCh}}\). Spontaneous decay of \(I_{\text{CCh}}\) (desensitization of \(I_{\text{CCh}}\)) occurred \((n=5)\) (Fig. 5Aa). To determine the \(I-V\) relationship, we applied a ramp pulse from +100 to \(-100\) mV for 500 ms. The \(I-V\) relationship showed a typical doubly rectifying shape (Fig. 5Ab). In the presence of 20 μM diC8-PIP2 in the pipette solution, the desensitization of \(I_{\text{CCh}}\) (desensitization of \(I_{\text{CCh}}\)) occurred \((n=5)\) (Fig. 5Aa). To determine the \(I-V\) relationship, we applied a ramp pulse from +100 to \(-100\) mV for 500 ms. The \(I-V\) relationship showed a typical doubly rectifying shape (Fig. 5Ab). During desensitization, the \(I-V\) relationship showed a typical doubly rectifying shape (Fig. 5Bb). In control mouse ileal myocytes, the inward current spontaneously declined to 7.1 ± 3.1% of the initial peak amplitude after 2 min of CCh application \((n=5)\). In the presence of 20 μM diC8-PIP2 in the pipette solution, however, the inward current remained at 64 ± 5.1% of the initial peak amplitude 2 min after CCh application. There was no change in the activation process \((n=5)\) (Fig. 5C). These results suggest that TRPC5 is a candidate NSCC, activated by muscarinic receptor stimulation in gastrointestinal smooth muscle cells.
motif (Thr-Thr-Arg-Leu), which links TRPC4 to F-actin
cytoskeleton. Its inhibitory action was prevented by the application of
PKC inhibitor (Fig. 4B). We suggest that PKC might be the downstream
of TRPC5. In our previous study, the deletion of the C-terminal PDZ binding
target of PIP2, and that its inhibitory action was de-
slowly during CCh treatment. Dashed lines show zero current. b. F-V relationships showed a typical
duably rectifying shape. (C) CCh induced inward currents were normalized against the peak
amplitude and average values are plotted against time (■ control; ○, intracellularly ap-
pplied PIP2). Asterisks indicate significant differences (* p<0.05).

**DISCUSSION**

We have demonstrated that intracellularly-applied PIP2 attenuated the desensitization of TRPC5 currents. We have also shown that the actin cytoskeleton plays a role in the activation and the desensitization of these currents. Moreover, we have shown that in mouse ileal myocytes, the desensitization of these currents is dependent upon PKC phosphorylation of TRPC5 channel. 

The effect of CaM and MLCK on murine ileal myocytes was studied. CaM and MLCK modulated the activation of rat TRPC5. We determined that attenuation of the desensitization of TRPC5 current was specific to PIP2, as other PIs had no effect. This indicates that the effects are not simply a negative charge effect, as in the case of activation of the TRPC4 current.

Fig. 6. The Desensitization of CCh-Activated Inward Current and Its I–V Relationship Recorded from Murine Ileal Myocytes Using the Whole-Cell Patch-Clamp Technique

(A) a. Whole cell currents were recorded under the condition of 140 mM [Cs+], CCh (50 μM) induced an inward current, which decayed spontaneously during CCh treatment. Slow ramp depolarizations from +100 to –100 mV were applied at a holding potential of –60 mV. Dashed lines show zero current. b. I–V relationships showed a typical doubly rectifying shape. (B) b. In the presence of 20 μM diC8-PIP2 in the pipette solution, CCh (50 μM) induced an inward current, which decayed slowly during CCh treatment. Dashed lines show zero current. b. I–V relationships showed a typical doubly rectifying shape. (C) CCh induced inward currents were normalized against the peak amplitude and average values are plotted against time (■ control; ○, intracellularly applied PIP2). Asterisks indicate significant differences (* p<0.05).

**REFERENCES**
