Desensitization of canonical transient receptor potential channel 5 by protein kinase C

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Desensitization of canonical transient receptor potential channel 5 by protein kinase C. Am J Physiol Cell Physiol 289: C591–C600, 2005. First published April 20, 2005; doi:10.1152/ajpcell.00440.2004.—The classic type of transient receptor potential channel (TRPC) is a molecular candidate for Ca2+-permeable cation channel in mammalian cells. TRPC5 is desensitized rapidly after activation by G protein-coupled receptor. Herein we report our investigation into the desensitization of mTRPC5 and localization of the molecular determinants of this desensitization using mutagenesis. TRPC5 was initially activated by muscarinic stimulation using 100 nM carbachol (CCh) and then decayed rapidly even in the presence of CCh (desensitization). Increased EGTA or omission of MgATP in the pipette solution slowed the rate of this desensitization. The protein kinase C (PKC) inhibitors, 1 μM chelerythrine, 100 nM GFI09203X, or PKC peptide inhibitor (19–36), inhibited this desensitization of TRPC5 activated by 100 μM CCh. When TRPC5 current was activated by intracellular GTPγS, PKC inhibitors prevented TRPC5 desensitization and the mutation of TRPC5 T972 to alanine slowed the desensitization process dramatically. We conclude that the desensitization of TRPC5 occurs via PKC phosphorylation and suggest that threonine at residue 972 of mouse TRPC5 might be required for its phosphorylation by PKC.

...nonselective cation channels; Ca2+-permeable cation channels

The classic type of transient receptor potential channel (TRPC) is a molecular candidate for Ca2+-permeable cation channel in mammalian cells. It consists of seven types of TRPC. TRPC3, TRPC6, and TRPC7 belong to one group and are activated by diacylglycerol (DAG), whereas TRPC1, TRPC4, and TRPC5 belong to another and are not activated by DAG. Moreover, there are two types of Ca2+-permeable cation channels. Store-operated channels (SOC) are activated by store depletion, whereas receptor-operated channels (ROC) are activated by the stimulation of G protein-coupled receptors (GPCRs). TRPC4 is activated both by store depletion (36) and by GPCR stimulation (26, 30, 31, 38), whereas TRPC5 was initially suggested as a SOC (25). However, after the initial report, TRPC5 was shown to be activated by GPCR stimulation and was suggested to be an ROC (10, 12, 22, 23, 30, 32, 35).

TRPC4 and TRPC5 are rapidly desensitized after activation by GPCR, and this desensitization does not depend on extracellular monovalent cations such as Na+ or Cs+. Under both monovalent cation conditions, TRPC4 and TRPC5 were found to be desensitized after the activation of muscarinic receptors. Even when intracellular GTPγS was used to activate TRPC4 and TRPC5, TRPC4 and TRPC5 currents were rapidly desensitized. Activated G proteins stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) through PLC-β, and PIP2 is hydrolyzed into inositol 1,4,5-trisphosphate (IP3) and DAG. This hydrolysis of IP3 was suggested to be associated with the inactivation of TRP melastatin 7 (TRPM7), although it activates other TRP channels such as TRPC3–TRPC7 and TRPV1. In the case of TRPCs, protein kinase C (PKC) was found to be involved in the desensitization process, whereas DAG activated TRPC3, TRPC6, and TRPC7 but not TRPC1, TRPC4, and TRPC5 (35). When 1-oleoyl-2-acetyl-sn-glycerol, a membrane-permeable analog of DAG, was applied before GPCR stimulation by acetylcholine, it inhibited TRPC5 activation (35). Intracellular Ca2+ concentration ([Ca2+]i) measurements showed that PKC inhibited all TRPC channels, whereas DAG activated TRPC3, TRPC6, and TRPC7. We have been studying the desensitization mechanism of TRPC5 using whole cell patch-clamp techniques. In our previous studies (12, 38), when we increased the extracellular Ca2+ concentration ([Ca2+]o) from 0 to 10 mM, it caused initial facilitation and a subsequent faster desensitization; when intracellular GTPγS was used to activate TRPC4 and TRPC5, a similar phenomenon was observed. These results suggest that Ca2+-dependent processes are involved in the desensitization of TRPC5.

We recorded TRPC5 current electrophysiologically and used characteristic current-voltage (I-V) relationships as markers of TRPC5 expression (12, 38; see also Ref. 5). The desensitization of TRPC5 by PKC was found to be dependent on extracellular and intracellular Ca2+. intracellular MgATP or EGTA, and PKC inhibitors. To identify the PKC phosphorylation sites responsible for the desensitization, we mutated 11 putative PKC phosphorylated TRPC5 sites. Seven sites had no or only a slight effect on the desensitization process, whereas mutations of the other three sites caused TRPC5 not to be expressed on the plasma membrane. However, only the T972A mutant slowed the desensitization process.

Experimental Procedures

Molecular biology. Plasmids containing mouse TRPC5 or TRPC4 were kindly donated by Dr. M. Schaefer. Point mutations in TRPC5 or TRPC4 were introduced using a QuickChange site-directed mutagenesis kit (Stratagene) with appropriate primer sets. To produce...
deletion mutants of the last six amino acids of TRPC5 (QVTTRL) or TRPC4 (YVTTRL), glutamine (CAA, TRPC5) or tyrosine (TAT, TRPC4) was replaced with a stop codon (TAA). Mutant sequences were confirmed using DNA sequencing.

Cell culture and transient transfection. Human embryonic kidney (HEK)-293 cells (American Type Culture Collection, Manassas, VA) were maintained according to the supplier’s recommendations. For transient transfection, cells were seeded in 12-well plates. The next

Fig. 1. Effect of intracellular EGTA concentration ([EGTA]i) on mouse transient receptor potential channel 5 (mTRPC5) desensitization. Whole cell currents were recorded under conditions of 0.5 mM [EGTA], (A), 1 mM [EGTA], (B), or 2 mM [EGTA], (C) using the patch-clamp technique. When 100 μM carbachol (CCh) was applied at a holding potential of −60 mV, an inward current was activated in TRPC5-expressing human embryonic kidney (HEK)-293 cells (a). The degree of TRPC5 desensitization depended on [EGTA]. Dotted lines indicate zero currents. (1) and (2) indicate ramp pulses before and during the application of CCh, respectively. To obtain current-voltage (I-V) relationships, we applied a ramp pulse from +100 to −100 mV for 1 s. The I-V relationship of TRPC5 had a doubly rectifying shape (b). The slope between 0 and +50 mV seemed to depend on [EGTA]. D: degree of desensitization. Desensitization scores (I_{100s}/I_{max}) were found to depend on [EGTA]. *P < 0.05.
day, 0.5–2 μg/well of pcDNA vector containing the cDNA of TRPC5 or point mutants of TRPC5 were mixed with 50–100 ng/well of pEGFP-C1 (Clontech) and transfected into cells using FuGENE 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer’s protocol. After 18–24 h, cells were trypsinized and used for whole cell recordings.

Electrophysiology. Whole cell currents were recorded using an Axopatch 200A amplifier (Axon Instruments). Currents were filtered at 5 kHz (-3 dB, 4-pole Bessel), digitized using a Digidata 1200 Interface (Axon Instruments), and analyzed using a personal computer equipped with pClamp 9.0 software (Axon Instruments). Patch pipettes were made from borosilicate glass and had resistances of 3–6 MΩ when filled with standard intracellular solutions. For whole cell experiments, we used an external bath medium (normal Tyrode solution) of the following composition (in mM): 135 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 glucose, and 10 N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] (HEPES), with pH adjusted to 7.4 using NaOH. Cs⁺-rich external solution was made by replacing NaCl and KCl with equimolar CsCl. CaCl2 was simply omitted from the external bath medium to produce Ca²⁺-free PSS. The pipette solution contained (in mM) 140 CsCl, 10 HEPES, 0.2 Tris-GTP, 0.5 EGTA, and 3 MgATP, with pH adjusted to 7.3 using CsOH. The calculated junction potential between the pipette and bath solutions used for all cells during sealing was 4 mV (pipette negative, using pClamp 9.0 software). No junction potential correction was applied. Experiments were performed at room temperature (18–22°C). Gravity was used to superfuse the solution and apply the drugs. The chamber volume was 400 μl, and the time required to reach the chamber was ~30 s. Latency was the time from arrival time of solution to the chamber to the peak activation of current.

Statistics. Results are expressed as means ± SE. Where appropriate, results were compared using Student’s t-test.

RESULTS

Effects of intracellular EGTA and MgATP on mTRPC5 desensitization. Whole cell currents were recorded using patch-clamp techniques. Initially, whole cell currents were recorded under the condition of normal Tyrode solution (140 mM extracellular Na⁺ concentration, [Na⁺]o, and intracellular Cs⁺ concentration, [Cs⁺]i). To obtain I-V relationships, we applied a ramp pulse from +100 to -100 mV for 1 s. After changing the external solution from normal Tyrode to 140 mM [Cs⁺]o, solution, basal currents increased slightly because of the constitutive activity of TRPC5. Sometimes these currents were activated up to 1 nA without any stimulation of TRPC5 by carbachol (CCh), and therefore we usually waited for at least 2 min before adding CCh. Whole cell currents also were recorded under the condition of 140 mM extracellular Cs⁺ concentration ([Cs⁺]o) and [Cs⁺], as a control to obtain the I-V relationship of mTRPC5 activated by CCh by subtraction. When 100 μM CCh was added at a holding potential of -60 mV in the presence of 0.5 mM intracellular EGTA concentration ([EGTA]), an inward current was activated (Fig. 1Aa). Latency to activation was 38 ± 4 s (n = 11). The I-V relationship, obtained by subtracting the current observed in the absence of CCh from that in the presence of CCh, showed a typical doubly rectifying shape (Fig. 1Ab). We used only the results obtained from cells producing the typical I-V relationship of TRPC5.

The TRPC5 current activated by stimulating muscarinic receptors decayed spontaneously to the basal level even during the first application (Fig. 1A), a process referred to as desensitization, and the degree of this desensitization varied among
cells. We defined the desensitization score as the ratio of the current at 100 s to peak current. Accordingly, desensitization scores were 0.06 ± 0.01 (n = 11) under the control condition of 0.5 mM [EGTA].

When we increased the concentration of EGTA in the pipette solution, latency to activation increased and the rate of desensitization slowed (Fig. 1, B and C). This latency increased to 128 ± 18 s (n = 10) at 1 mM [EGTA], and 123 ± 10 s (n =
Desensitization scores increased to 0.96 ± 0.08 (n = 12) for treatment with GF109203X (Fig. 5D). With intracellular GTPγS, the TRPC5 current increased, and it decayed upon changing the external 140 mM Na⁺ solution to 140 mM Cs⁺ solution (n = 10). Latency to activation was 177 ± 21 s (n = 6). When we pretreated cells with GF109203X, latency increased and the TRPC5 current did not then decay rapidly by changing the external 140 mM Na⁺ solution to 140 mM Cs⁺ solution. The latency increased to 218 ± 22 s (n = 12) after pretreatment with GF109203X, and desensitization scores increased to 0.91 ± 0.11 (n = 6) after GF109203X pretreatment (Fig. 5D). These results provide evidence that PKC is involved in the desensitization process and that the PKC action site is downstream of the G protein. There are two possibilities: either TRPC5 is desensitized by PKC phosphorylation, or other proteins such as PLC or postsynaptic density-95/Drosophila disk large/zonula occludens-1 homology (PDZ) domain-containing proteins involved in the signaling process are desensitized.

Effects of PKC inhibitors on the desensitization of mTRPC5. Thus we searched for putative PKC phosphorylation sites and mutated serine or threonine to alanine. There are 14 putative PKC phosphorylation sites in mTRPC5. Three residues, S351, S451, and S490, exist in the transmembrane domains, and 11 residues are located on the cytoplasmic side (Fig. 6A). We mutated serine or threonine at these 11 residues to alanine and then investigated whether these changes altered desensitization when CCh was applied. The mutation of T972 to alanine slowed the activation and desensitization processes to the greatest extent (Fig. 6B): latency increased to 200 ± 14 s (n =

8) at 2 mM [EGTA]. Under the control condition of 0.5 mM [EGTA], the current decayed to one-half of its peak value at 36 s after CCh application, and this time required to reach half peak value increased to 225 s at 1 mM [EGTA]. No desensitization was observed with 2 mM [EGTA] in the pipette. Desensitization data increased from 0.06 ± 0.01 (n = 11) under the control condition of 0.5 mM [EGTA], to 0.59 ± 0.02 (n = 10) at 1 mM [EGTA], and 0.91 ± 0.03 (n = 8) at 2 mM [EGTA] (Fig. 1D), suggesting that the desensitization process depends on intracellular Ca²⁺. However, when we increased [EGTA] to 10 mM, no TRPC5 currents showing typical I-V relationships were observed. In addition, CCh could not activate TRPC5 current after pretreatment with 10 μM cyclopiazonic acid (CPA) or 30 μM 2,5-di-(t-butyl)-1,4-hydroquinone (BHQ), and when 10 μM CPA or 30 μM BHQ were applied alone to deplete Ca²⁺ stores, TRPC5 current was not induced either. Thus, to activate TRPC5 by muscarinic stimulation, optimal [Ca²⁺] levels are needed.

When we omitted intracellular MgATP, latency to activation increased and the rate of desensitization slowed (Fig. 2A), and latency to activation was 63 ± 9 s (n = 10). During the application of CCh, the TRPC5 current did not decay (n = 6). Desensitization scores increased to 0.96 ± 0.01 (n = 11) under the MgATP-free pipette condition (Fig. 2B). These results suggest that the desensitization process depends on the phosphorylation of intracellular TRPC5.

Effects of PKC inhibitors on the desensitization of mTRPC5. Because desensitization depended on [Ca²⁺] and MgATP, we investigated whether PKC is involved in the desensitization process (Fig. 3). Chelerythrine and GF109203X (both PKC inhibitors) increased latency to activation and slowed desensitization. Latency to activation increased from 38 ± 4 s (n = 11) at control to 251 ± 45 s (n = 8) at 100 nM GF109203X and 216 ± 41 s (n = 7) at 1 μM chelerythrine. TRPC5 currents decayed rapidly during the first application of CCh. When we pretreated cells with the PKC inhibitor, 100 nM GF109203X, TRPC5 currents activated slowly (n = 5). Chelerythrine (1 μM) had an effect similar to that of 100 nM GF109203X (n = 5). Desensitization data increased from 0.06 ± 0.01 (n = 11) under the control condition of 0.5 mM [EGTA], to 0.95 ± 0.04 (n = 8) after treatment with GF109203X and to 0.94 ± 0.06 (n = 7) after treatment with chelerythrine (Fig. 3D). The internal effect of PKC peptide inhibitor (19–36) (Calbiochem) was also investigated (Fig. 4) and was found to slow the desensitization process; desensitization data increased to 0.81 ± 0.07 (n = 4).

When we applied 100 μM CCh 3 min after the first application of CCh, the current was activated less than it had been during the first application. The current amplitude decreased from 1.25 nA to 100 pA at +100 mV and from -650 pA to 60 pA at -100 mV (Fig. 5A). When we pretreated cells with the PKC inhibitor, 100 nM GF109203X, latency to activation increased and the current did not decay during the second application of CCh. However, the I-V relationships for the first and second applications of CCh were similar (Fig. 5B). Even when we activated TRPC5 current with intracellular GTPγS, PKC inhibitor prevented the desensitization (Fig. 5C). In this experiment, we compared the peak amplitude of the first application (I_max1) with that of the second application (I_max2) and used I_max2/I_max1 as a desensitization score. Desensitization scores increased from 0.07 ± 0.01 (n = 9) for the control to

Fig. 4. Effect of PKC peptide inhibitor on mTRPC5 desensitization. A: whole cell currents were recorded under the condition of symmetrical 140 mM [Ca²⁺] and 140 mM [Cs⁺], using patch-clamp techniques. The pipette contained 10 μM PKC peptide inhibitor (Calbiochem). When 100 μM CCh was applied at a holding potential of −60 mV, an inward current was activated in TRPC5-expressing HEK-293 cells. Moreover, this current did not decay during the continuous application of CCh. Dotted line indicates zero current. (1) and (2) represent ramp pulses during the first and the second CCh applications, respectively. B: to obtain I-V relationships, we applied a ramp pulse from +100 mV to −100 mV for 1 s. (1) and (2) show I-V curves obtained by applying the ramp pulses in A.
Fig. 5. Effect of PKC inhibitor on mTRPC5 desensitization. A: whole cell currents were recorded under the condition of symmetrical 140 mM [Cs⁺]/140 mM [Cs⁺] using patch-clamp techniques. When 100 μM CCh was applied at a holding potential of −60 mV, an inward current was activated in TRPC5-expressing HEK-293 cells. Dotted line indicates zero current (a). To obtain I-V relationships, we applied a ramp pulse from +100 to −100 mV for 1 s. The I-V relationship shows a doubly rectifying shape (b). (1) and (2) represent ramp pulses before and during the application of CCh, respectively. B: pretreatment with PKC inhibitor, 100 nM GF109203X, inhibited TRPC5 current desensitization. No difference was observed between I-V relationships in the absence or presence of PKC inhibitor. C: effect of PKC inhibitor on the desensitization of mTRPC5 by intracellular GTPγS. Pretreatment with the PKC inhibitor, 100 nM GF109203X, inhibited TRPC5 current desensitization by intracellular GTPγS. D: degree of desensitization (I_{10s}/I_{max}). This desensitization was inhibited by PKC inhibitor treatment, regardless of activation with CCh or intracellular GTPγS. *P < 0.05.
Fig. 6. Desensitization of TRPC5 mutants. A: schematic diagram of mTRPC topology. Putative PKC phosphorylation sites are shown. B: whole cell current in the T972A mutant. Whole cell currents were recorded under the condition of symmetrical 140 mM [Cs⁺], and 140 mM [Cs⁺], using patch-clamp techniques. When 100 μM CCh was applied at a holding potential of −60 mV, an inward current was activated in TRPC5-expressing HEK-293 cells. This current did not decay after the initial activation of TRPC5 by CCh. Dotted line indicates zero current (a). To obtain I-V relationships, we applied a ramp pulse from +100 to −100 mV for 1 s. The I-V relationship shows a doubly rectifying shape (b). (1) and (2) represent ramp pulses before and during the application of CCh, respectively. C: whole cell current in a S842A mutant, which shows TRPC5 desensitization. Dotted line indicates zero current (a). I-V relationships of all mutants in this figure show doubly rectifying shapes (b). D: degree of desensitization. Desensitization scores (I_{100s}/I_{max}) depend on mutation sites. The T972A mutation slowed the desensitization process to the greatest extent. *P < 0.05.
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15), and desensitization scores increased to 0.91 ± 0.04 (n = 15). The serine and threonine mutations to alanine had a smaller effect on desensitization in four mutants, T47A, S666A, S752A, and S833A, than in the T972A mutant (Fig. 6D). Desensitization scores were 0.09 ± 0.02 (n = 9), 0.16 ± 0.02 (n = 5), 0.30 ± 0.03 (n = 7), and 0.18 ± 0.02 (n = 7) for the T47A, S666A, S752A, and S833A mutants, respectively (Fig. 6D). Mutation had no effect on desensitization for three mutants, T700A, S799A, and S842A (Fig. 6, C and D), with respective desensitization scores of 0.08 ± 0.01 (n = 7), 0.10 ± 0.02 (n = 6), and 0.10 ± 0.01 (n = 7) (Fig. 6, C and D). On the other hand, three mutants, S117A, S189A, and S261A (between the NH2 terminus and the first transmembrane domain) were not expressed in the plasma membrane, and we could not record any TRPC5 current for these three mutants. Moreover, these three sites are not located within putative ankyrin repeat domains or the putative coiled-coil region.

Deletion mutants of the last six amino acids of mTRPC4 (YVTTRL) and mTRPC5 (QVTTRL) were prepared and expressed in HEK-293 cells (Fig. 7) and were found not to produce currents [mTRPC4 (n = 20) and mTRPC5 (n = 21)]. Only one cell of each mutant produced a small current. The I-V relationship of the mTRPC5 mutant (∆QVTTRL) was similar to that of the wild type (Fig. 7B), but the I-V relationship of the mTRPC4 mutant (∆YVTTRL) differed (Fig. 7A). The I-V relationship of the mTRPC4 mutant (YVTTRL) resembled that of the D633N mutant of TRPC5 (21). The flat region of I-V relationship from 0 to −40 mV disappeared in the ∆YVTTRL mutant of mTRPC4.

DISCUSSION

In the present study, we have shown that TRPC5 itself might be phosphorylated at site T972 by PKC and desensitized. First, the desensitization process became slower when [Ca2+]o was decreased by Ca2+ buffer EGTA. Second, omitting intracellular MgATP slowed the desensitization process of TRPC5. Third, the PKC inhibitors GF109203X, chelerythrine, and PKC peptide inhibitor inhibited the desensitization of TRPC5. When we activated TRPC5 current with intracellular GTPγS, PKC inhibitor prevented TRPC5 desensitization. Fourth, the mutation of threonine at site 972 to alanine completely inhibited the PKC desensitization of TRPC5.

In Drosophila, PKC is involved in the termination of light-induced response. The Drosophila TRP channel exists in a functional complex containing photoreceptor PLC, PKC, and calmodulin held within the PDZ domain-containing INAD (inactivation-no afterpotential D) scaffold protein (16, 17). There is evidence that the PKC within this complex directly phosphorylates and inhibits the TRP channel in a negative feedback loop that controls phototransduction (8, 14). In vertebrate systems, TRPC channels may be organized within similar regulatory complexes via PDZ domain-containing proteins such as the Na+/H+ exchanger regulatory factor (NHERF), which is known to interact with and organize TRPC4 and TRPC5 channels and PLC-β isofoms (33). The mutation site T972 resides in the VTTRL motif and binds to the PDZ domain. PKC phosphorylation of T972 might induce TRPC5 desensitization by disrupting the interaction motif of TRPC5 (TTRL) with the PDZ domain of NHERF or other PDZ domain-containing proteins. Such disruption causes TRPC5 to

Fig. 7. The expression of the deletion mutant of TRPC5 (∆QVTTRL) or TRPC4 (∆YVTTRL). A: whole cell current in a deletion mutant ∆YVTTRL of mTRPC4. Whole cell currents were recorded under the condition of symmetrical 140 mM [Cs+]o and 140 mM [Cs+]i using patch-clamp techniques. When 100 μM CCh was applied at a holding potential of −60 mV, a small inward current was activated in TRPC4-expressing HEK-293 cells. Dotted line indicates zero current (a). To obtain I-V relationships, we applied a ramp pulse from +100 to −100 mV for 1 s. The flat region of the I-V relationship from 0 to +40 mV disappeared. (1) and (2) represent ramp pulses before and during the application of CCh, respectively (b). B: whole cell current in deletion mutant ∆QVTTRL of mTRPC5 activated by intracellular GTPγS (0.2 mM) under the condition of symmetrical 140 mM [Cs+]o and [Cs+]i, using patch-clamp techniques. To obtain I-V relationships, we applied a ramp pulse from +100 to −100 mV for 1 s. I-V relationship shows a doubly rectifying shape.
be disconnected from NHERF, PLC-β, or the cytoskeleton and to be desensitized via the endocytosis or degradation of TRPC5. The mutation of T972 to alanine might effect desensitization by maintaining the interaction motif of TRPC5 (TTRL) with the PDZ domain of NHERF or other PDZ domain-containing proteins. There is also evidence that PDZ-ligand interactions are disrupted by phosphorylation, which typically occur on COOH-terminal amino acids. These interactions were disrupted by PKC phosphorylation in the GluR2 receptor (4) or by PKA phosphorylation in stargazin (2). It is equally possible that PKC phosphorylates the PDZ domain of NHERF or other PDZ domain-containing proteins that interact with the PDZ binding motif of TRPC5 (TTRL). Although less prevalent than phosphorylation of the COOH-terminal motif, PDZ-peptide interactions also can be regulated by the phosphorylation of the PDZ domain. Ca²⁺/calmodulin-dependent protein kinase II (CaMKII)-dependent phosphorylation of the synapse-associated protein 97 (SAP97) PDZ1 domain disrupted the interaction between SAP97 and the NMDA receptor 2A subunit, but not with the glutamate receptor type 1 (GluR1) AMPAR [α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor] subunit (7).

The PDZ-interacting domain of TRPC4, which belongs to the same subfamily as TRPC5, controls TRPC4 localization and surface expression in transfected HEK-293 cells (15). Moreover, TRPC4 mutant lacking the TRL motif accumulated in cell outgrowth and exhibited a distribution unlike that of the wild-type channel, and deletion of the TRL motif reduced the amount of channel associated with the plasma membrane. According to our results (Fig. 7), ΔYVTTRL mutant of mTRPC4 or ΔQVTTRL mutant of mTRPC5 is not expressed well at the plasma membrane. To the contrary, ΔQVTTRL mutants of rat TRPC5 showed properties similar to those of the wild-type rat TRPC5 (20). ΔQVTTRL mutants of rat TRPC5 were expressed well and showed PKC desensitization, although the desensitization process seemed a little slower than that of the wild-type form. In mouse TRPC4 and TRPC5, the PDZ domain-binding motif might also be important for controlling TRPC localization and surface expression in transfected HEK-293 cells.

In gastric guinea pig myocytes, a nonselective cation channel was activated by muscarinic stimulation. Desensitization of this channel was found to depend on PKC activity (11), to be dependent on [Ca²⁺], and to be reversed by PKC inhibitors. Conventional PKC was involved in the desensitization process. When [Ca²⁺] was increased to 200 nM, PKC-α was activated and induced desensitization. However, at <200 nM [Ca²⁺], PKC-α was not activated and the current was not desensitized. We also have shown that TRPC5 is a molecular candidate for the nonselective cation channel that is activated by muscarinic stimulation in murine gastric myocytes (12, 38). The desensitization of TRPC5 might correspond to the desensitization of the nonselective cation channels activated by muscarinic stimulation in native tissues, such as in the gastric myocytes of guinea pigs and murine gastric myocytes.

On the other hand, PKC sensitizes TRPV1 channel activity by augmenting the channel open probability (1, 27). Furthermore, PKC-mediated phosphorylation also reduces the heat threshold of channel activation (27, 34). Thus PKC sensitizes TRPV1 and induces inflammatory hyperalgesia. A recent report showed that PKC sensitizes TRPV1 in association with the rapid recruitment of vesicular channels to the cell surface by regulated exocytosis (18). TRPV1 also exhibits a time- and Ca²⁺-dependent desensitization, a long-lasting refractory state during which the receptor does not respond to vanilloids or to other stimuli (9). The association of the COOH terminus of TRPV1 with PIP2 inhibits TRPV1 channel activity (3, 28), and its interaction with calmodulin promotes channel desensitization (19). Although PIP2 hydrolysis activates both TRPV1 and TRPC5, their desensitization processes differ. In TRPC5, PKC phosphorylation is involved in desensitization, whereas calmodulin is involved in the desensitization of TRPV1.

The PKC-mediated inhibition of receptor-induced PLC provides an important feedback loop mediated by DAG and Ca²⁺ on PLC enzyme (6, 13, 29, 37). If this process is involved in the desensitized process, all TRPC5 mutants should have been desensitized. However, the T972A mutant was not desensitized. This result suggests that TRPC5 itself might become phosphorylated by PKC and desensitized. In addition, pretreatment with PKC inhibitor should activate TRPC5 if PKC-mediated inhibition of receptor-induced PLC provides an important feedback loop mediated by DAG and Ca²⁺ on PLC enzyme. To the contrary, pretreatment with PKC inhibitor did not have any effect at lower concentrations and sometimes blocked TRPC5 current activation at a concentration >1 μM.

We conclude that the desensitization of TRPC5 occurs via PKC phosphorylation and that threonine at residue 972 might be important for the PKC phosphorylation of mTRPC5, although direct phosphorylation of the mTRPC5 channel itself was not shown.

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