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Complex Functions of Phosphatidylinositol 4,5-bisphosphate in Regulation of TRPC5 Cation Channels

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Abstract

The canonical transient receptor potential (TRPC) proteins have been recognized as key players in calcium entry pathways activated through phospholipase C-coupled receptors. While it is clearly demonstrated that members of the TRPC3/6/7 subfamily are activated by diacylglycerol, the mechanism by which phospholipase C activates members of the TRPC1/4/5 subfamily remains a mystery. Here, we provide evidence for both negative and positive modulatory roles for membrane polyphosphoinositides in the regulation of TRPC5 channels. Depletion of polyphosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate (PIP₂) through inhibition of phosphatidylinositol 4-kinase activates calcium entry and membrane currents in TRPC5-expressing, but not in TRPC3- or TRPC7-expressing cells. Inclusion of polyphosphatidylinositol-4-phosphate or PIP₂, but not phosphatidylinositol-3,4,5-trisphosphate, in the patch pipette inhibited TRPC5 currents. Paradoxically, depletion of PIP₂ with a directed 5-phosphatase strategy inhibited TRPC5. Furthermore, when the activity of single TRPC5 channels was examined in excised patches, the channels were robustly activated by PIP₂. These findings indicate complex functions for regulation of TRPC5 by PIP₂, and we propose that membrane polyphosphoinositides may have at least two distinct functions in regulating TRPC5 channel activity.

Keywords

ion channels; non-selective cation channels; TRPC5 channels; phosphatidylinositol 4,5-bisphosphate; phospholipase C

Introduction

Calcium signaling in many cell types is initiated by stimulation of membrane receptors coupled to polyphosphoinositide-specific phospholipase C (PLC). The action of PLC on the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP₂) leads to the formation of two second messengers: inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ activates IP₃ receptor channels in the endoplasmic reticulum and causes release of intracellular stored Ca²⁺ [1]. The fall in Ca²⁺ content within the lumen of the endoplasmic reticulum then signals the subsequent activation of plasma membrane calcium-permeable channels, a process known

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as capacitative Ca^{2+} entry (CCE) or store-operated Ca^{2+} entry [2,3,4]. Although CCE seems to be present ubiquitously in receptor-regulated non-excitable cells, a wide variety of Ca^{2+} permeable channels are activated downstream of the PLC pathway by mechanisms not directly related to store depletion. Examples include channels activated by mechanisms involving IP_3 , cytosolic Ca^{2+} , DAG, cyclic nucleotides, phosphatidylinositol 3,4,5-trisphosphate (PIP_3), and arachidonic acid (for review see [5,6]). During the last decade, several investigations have implicated the members of the transient receptor potential (TRP) family of channel proteins as candidates for some of these Ca^{2+} entry pathways [6,7,8,9,10,11].

TRP proteins constitute a large superfamily of ion channels that are expressed in many tissues and cell types [12]. TRP channels are activated by a wide variety of external stimuli ranging from temperature and oxidative stress to hormones, growth factors and neurotransmitters [10,7,13]. TRP channels are cation permeable channels and are now fully recognized as key players in the maintenance of ion homeostasis and in cell signaling pathways. The TRP superfamily can be divided into three major families: (i) channels homologous to the vanilloid receptor called TRPV, that are activated by a variety of signals, including vanilloid compounds such as capsaicin, noxious signals, hypotonic cell swelling and heat; TRPV family consists of six Ca^{2+} -permeable channels [14,7,15,16,17]; (ii) the Melastatin-related TRP channel family (TRPM) containing eight members, with diverse functional properties such as mediating Ca^{2+} influx, controlling Mg^{2+} entry, modulating the membrane potential, and sensing cold and menthol in sensory neurons [18,19,20,13]; (iii) the canonical TRP family (TRPC), named for being structurally the closest to the superfamily founder, *Drosophila* TRP, are all activated through PLC-coupled receptors and were therefore proposed to encode components of native PLC-coupled channels, such as store-operated channels and second messenger-operated channels [8,21,11].

The TRPC family contains seven members (TRPC1 through 7). TRPC2 is a pseudogene in humans and is involved in pheromone sensing in other mammals [22]. TRPC3/6/7 represents a subfamily within TRPC family whose members can be activated by diacylglycerol produced by PLC action on PIP_2 [23,24,25,26,27]. However, the mechanism of activation *via* PLC-coupled receptors of members of the TRPC1/4/5 subfamily is still unclear. These channels are believed to play important roles in diverse physiological functions, for example in regulation of vascular tone [28] and in neurite outgrowth [29]. While most investigators agree that PLC is necessary for receptor activation of this group, the general finding is that neither IP_3 nor DAG activates these channels [27,30]. Otsuguro *et al.* [31] suggested that breakdown of PIP_2 , in conjunction with inputs from G-proteins and $[\text{Ca}^{2+}]_i$, leads to activation of TRPC4. In the current study, we have investigated the role of inositol lipids in regulation and activation of TRPC5. Consistent with the findings of Otsuguro *et al.* for TRPC4, we show that in intact cells, pharmacological depletion of phosphatidylinositol 4-phosphate (PIP) and PIP_2 specifically activates TRPC5 channels while inclusion of these polyphosphoinositides in the patch pipette inhibits TRPC5 currents. However, depletion of PIP_2 by a targeted 5-phosphatase inhibited TRPC5 activity, and in excised patches, PIP_2 activated TRPC5 channels. These data suggest that PIP and/or PIP_2 exert multiple effects on TRPC5.

Material and Methods

Reagents

Methacholine, Wortmannin, LY294002 and Rapamycin were purchased from Calbiochem. D (+)-sn-1,2-di-O-octanoylglycerol, 3-O-phospholinked (DiC8) polyphosphoinositides, PIP, PIP_2 and PIP_3 were purchased from Echelon Biosciences Incorporated.

Cell culture and cell lines

Human embryonic kidney (HEK293) cells were obtained from ATCC and were transfected, using Lipofectamine 2000 reagent (Invitrogen) according to the vendor's instructions, with pcDNA3 vector containing the coding sequence of TRPC3 or TRPC5. Cells stably expressing hTRPC3-green fluorescent protein (TRPC3-GFP) fusion protein used in this study were described earlier [26,32,33]. Cells stably expressing TRPC5 (murine TRPC5 cDNA was generously donated by Dr. Lutz Birnbaumer, National Institute of Environmental Health Sciences) were generated after transfection and subsequent selection by antibiotic resistance. Several clones were selected that gave robust Ca^{2+} entry signals in response to methacholine (for example, Figure 1A). Both TRPC3-GFP and TRPC5-expressing cell lines were grown under selection with G418 at 37 °C in Dulbecco's modified eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine in a humidified 95% air, 5% CO_2 incubator.

Ca^{2+} measurements

Ca^{2+} measurements were carried out with either Fura-2-AM or Fluo-4-AM and a cell imaging system (Intracellular Imaging, Inc., Cincinnati, OH) as described previously [34]. For Fura-2 measurements, the ratio obtained by dividing the emitted fluorescence from excitation at 340 nm by the emitted fluorescence measured after excitation at 380 nm is reported as an indicator of changes in intracellular Ca^{2+} concentration. For experiments using wortmannin, which is UV sensitive, Ca^{2+} measurements were carried out with the visible wavelength dye, Fluo-4. Briefly, cells were loaded with either 1 μM of Fura-2/AM (for 30 minutes) or 4 μM of Fluo-4/AM (for 1 hour), then washed and bathed in Hepes-buffered saline solution (HBSS; in mM, 140 NaCl, 4.7 KCl, 10 CsCl, 2 CaCl_2 , 1.13 MgCl_2 , 10 glucose, and 10 Hepes, pH 7.4) for at least 10 min before Ca^{2+} measurements are made. Basal cytosolic Ca^{2+} was determined in a field containing up to 100 cells and cytosolic Ca^{2+} was monitored after addition of enzyme inhibitors and/or receptor agonists.

Electrophysiology

Ion currents were recorded by using either the whole-cell or nystatin-patch configuration using an Axopatch-200B amplifier (Axon Instruments) as described previously [26]. The patch pipettes were pulled with a pipette puller (Sutter) from glass capillaries (Corning glass, 7052) and fire-polished. The finished pipettes had resistances ranging between 2–5 M Ω when filled with the following solution (for whole cell experiments, in mM): 140 CsCl, 10 BAPTA (cesium salt), 1 MgCl_2 , 2.2 CaCl_2 , and 10 Hepes, pH 7.2, with Tris base. In most experiments, this pipette solution was buffered to ~100 nM free Ca^{2+} calculated using WinmaxC software available from Stanford University website. For nystatin-patch experiments, the pipette contained (in mM): CsCl, 55; Cs_2SO_4 , 70; MgCl_2 , 1; CaCl_2 , 1; Glucose, 5; HEPES, 10; nystatin concentration: 200 $\mu\text{g}/\text{ml}$. The bath solution was (in mM): 140 NaCl, 4.7 KCl, 10 CsCl, 2.0 MgCl_2 , 10 glucose, 2.0 or 10 CaCl_2 , and 10 Hepes, pH 7.4, with NaOH. The osmolarity of these solutions was adjusted to 290–310 mOsm with glucose. Bath solution change was accomplished by gentle perfusion. Wild type HEK cells (HEK-Wt), TRPC3-GFP- and TRPC5-expressing HEK293 cells were plated the day before on coverslips. Before starting the experiments, coverslips were mounted in a perfusion chamber and electrophysiological measurements were performed at room temperature. An agar bridge served as the electrical connection between the bath and the signal ground. Whole cell currents were elicited by voltage stimuli lasting 250 ms, delivered every 5 seconds, with voltage ramps from –100 to +100 mV. Data were sampled at 5 kHz, filtered at 1 kHz, acquired with pCLAMP 9.0 software and analyzed with CLAMPFIT 9.0 (Axon).

For the single channel measurements in the inside-out configuration, the composition of the solutions present in the bath and the pipette (resistance varying between 3–6 M Ω) were

respectively (in mM): KCl, 120; HEPES, 20; EGTA, 10; CaCl₂, 4.1; MgCl₂, 1; pH 7.3 adjusted with KOH; and KCl, 140; HEPES, 10; CaCl₂, 1; MgCl₂, 1; pH 7.3 adjusted with KOH. Signals were low-pass filtered at 2 kHz, digitized at 20 kHz and then analyzed using the Clampfit 9.2 software (Axon Instr., USA).

Depletion of PIP₂ levels using Rapamycin

PIP₂ depletion was achieved by targeting of the type IV phosphatidylinositol 5-phosphatase (PI5-phosphatase) to the plasma membrane [35]. HEK293 Cells expressing TRPC5 were transfected with either a construct (2.5 µg) encoding a membrane targeted FRB domain of mTOR tagged with monomeric red fluorescent protein (mRFP), or with a construct (2.5 µg) encoding a modified cytoplasmic type IV 5-phosphatase fused to FKBP12 and tagged with mRFP, or co-transfected with both constructs. The plasma membrane targeted FRB domain of mTOR can be heterodimerized with FKBP12-5-phosphatase using rapamycin (100 nM) as described previously [35].

Results

Activation of TRPC5 by PLC-coupled receptors

An HEK293 cell line stably expressing TRPC5 channels (described in the Methods section) was used throughout this study. After activation with the muscarinic agonist, methacholine, TRPC5-expressing cells exhibited a Ca²⁺ entry pathway that was not found in the wild type cells. As described earlier, when used at concentrations ranging between 1 and 5 µM, gadolinium (Gd³⁺) is a potent inhibitor of CCE in HEK293 cells [32,36]. As expected, methacholine-activated CCE in HEK-Wt was completely blocked by 5 µM Gd³⁺ (Figure 1A). As previously shown for TRPC3 [32] and TRPC7 [37], TRPC5-expressing cells exhibited a receptor-activated Ca²⁺ entry pathway that was Gd³⁺-insensitive (Figure 1A). However, TRPC5 differs from members of the TRPC3/6/7 subfamily in two significant ways. First, TRPC5 channels were shown previously to be activated by higher concentrations of Gd³⁺ (50–100 µM) [38] while TRPC3 is inhibited by lanthanides at these high concentrations [39]. These results were confirmed in the present study and we found that TRPC5-expressing HEK cells show a Ca²⁺ entry in response to exogenous addition of 50 µM Gd³⁺ (Figure 1B). Second, the DAG analog, OAG (100 µM) fully activated TRPC3 while it failed to activate TRPC5 channels (Figure 1C), in confirmation of the findings of Venkatachalam et al. [27]. Furthermore, application of OAG to TRPC5-expressing HEK293 cells in the presence of the PKC inhibitor GF-109203X (10 µM) also failed to activate TRPC5 channels (data not shown).

PI-4K inhibitors (wortmannin and LY294002) specifically activate TRPC5 but not TRPC3 channels

Since neither second messenger (IP₃ or DAG) produced after PLC breakdown of PIP₂ appears to be involved in the activation of TRPC5 channels ([40,27], this study), we asked whether PIP₂ breakdown itself might be responsible for TRPC5 activation, for example by relieving tonic PIP₂ inhibition of the channels. To test this hypothesis, we first used a pharmacological strategy to deplete polyphosphoinositides in the cells.

The membrane permeant drugs, wortmannin and LY294002 are known to inhibit the phosphatidylinositol 3-kinase when used at relatively low concentrations (~100 nM and 10 µM respectively). When used at higher concentrations (20 µM for wortmannin and 100 µM for LY294002), these drugs are known to inhibit the phosphatidylinositol 4-kinase (PI4-K) [41,42]. PI4-K catalyses the phosphorylation of phosphatidylinositol (PI) into phosphatidylinositol 4 monophosphate (PIP), which in turn is converted into the substrate of PLC, PIP₂ via the action of the enzyme phosphatidylinositol 4-phosphate 5-kinase. Therefore,

efficient inhibition of PI4-K leads to subsequent PIP and PIP₂ depletion [41,42,43,44,45,46, 47].

As shown in Figure 2A, treatment of TRPC5-expressing HEK293 cells with 20 μ M wortmannin activates calcium entry in these cells while no effect of wortmannin was observed in wild type cells. Similar results were obtained using the drug LY294002 (Figure 2B) suggesting that this calcium entry is directly associated with TRPC5 channels and not to non-specific effects of these drugs. Concentrations of these drugs which inhibit phosphoinositide 3-kinase, but not PI4-K (10 μ M LY294002, 200 nM wortmannin) did not activate entry (data not shown). When LY294002 was applied to TRPC5-expressing cells in the continuous presence of Ca²⁺, there was a delay of about a minute before Ca²⁺ entry ensued (Figure 3A), as expected if this drug must penetrate the plasma membrane, inhibit PI4-K and cause depletion of polyphosphoinositides. Note that LY294002 caused a small release of Ca²⁺ (Figure 2B) that was not seen with wortmannin (Figure 2A). However, both drugs induced similar Ca²⁺ entry in TRPC5 cells. In addition, we also utilized another structurally dissimilar PI4-K inhibitor, phenylarsine oxide [48,44]; this agent caused a large release of Ca²⁺ stores, and thus was not employed in further experiments. However, we did note that, like wortmannin and LY294002, phenylarsine oxide activated Gd³⁺-insensitive Ca²⁺ entry in TRPC5-expressing cells, and not in wild-type or TRPC3-expressing cells (see below).

The effects of wortmannin and LY294002 are specific to TRPC5 channels. An HEK293 cell line expressing TRPC3 channels showed no increase in calcium entry after application of 100 μ M LY294002 (Figure 2C). Similar results were obtained with 20 μ M wortmannin (data not shown). Rather, receptor activation of TRPC3 channels in HEK293 cells was completely abrogated after treatment with 100 μ M LY294002 (Figure 2D), presumably because PIP₂ is necessary for TRPC3 activation as a source of DAG, the signal for TRPC3 activation. Similar results were obtained when TRPC3-expressing HEK293 cells were treated with 20 μ M wortmannin (data not shown). In addition, TRPC7 behaved identically to TRPC3; Ca²⁺ entry was not activated by PI4-K inhibitors, and agonist-activated Ca²⁺ entry was blocked by these drugs (data not shown). Note that in Figure 2D, a smaller but still significant mobilization of Ca²⁺ is seen, yet subsequent addition of Ca²⁺ shows essentially no TRPC3 activity. This is likely due to the fact that, in the presence of PI-4 kinase inhibitors, stimulation of phospholipase C-linked receptors yields a diminished production of phospholipase C products which is transient due to rapid run down of PIP₂ levels [49,42].

The experiments in Figure 2D indicate a diminished Ca²⁺ release response, which results from partial depletion of PIP₂ by LY294002 cells. We carried out a series of such measurements determining the magnitude of the transient Ca²⁺ release signal following methacholine addition in TRPC5-expressing cells as an indication of available PIP₂ levels. This transient response was significantly reduced in LY294002-treated cells, indicative of a reduction in available PIP₂ (Control, 0.86 ± 0.02 ratio units, n=184; LY294002, 0.49 ± 0.02 ratio units, n=206; P<0.0001). Although this is an indirect indicator of PIP₂, it has the advantage of providing information on PIP₂ specifically available for PLC-degradation. This information should be more meaningful than whole cell levels of polyphosphoinositides, which are known to reflect a number of pools of lipids, some not associated with the plasma membrane [50,51,52].

The plasma membrane calcium ATPase is known to be tightly regulated by PIP₂ [53,54,55]. Thus we considered that the rise in Ca²⁺ seen in cells treated with inhibitors of PI4-K might result, at least in part, from reduced Ca²⁺ extrusion. We examined the rate of Ca²⁺ extrusion from TRPC5-expressing cells after elevation of [Ca²⁺]_i by carbachol and by LY294002. However, there was no difference between these two stimuli in the rate of [Ca²⁺]_i decline following removal of extracellular Ca²⁺, indicating that the elevation in Ca²⁺ due to LY294002 does not result from a reduction in Ca²⁺ extrusion (Supplemental Figure).

Wortmannin and LY2942002 activate a current in TRPC5-expressing HEK cells similar to that activated by methacholine

We have shown that drugs that deplete polyphosphoinositides such as wortmannin and LY294002 activate a calcium entry pathway in TRPC5-expressing cells but not in TRPC3- or TRPC7-expressing cells or in wild type HEK293 cells. We next carried out patch-clamp current measurements initially in the perforated-patch configuration to determine if these drugs induce an ionic current in TRPC5-expressing HEK293 cells. As shown in Figure 3, the muscarinic-cholinergic agonist, carbachol, and both PI4-K inhibitors, activated inward and outward currents in TRPC5-HEK293 cells. None of these agents induced an increased current in non-transfected cells. The currents activated by carbachol and the PI4-K inhibitors were similar in that all three gave activation of larger inward than outward currents, and all three showed the double rectification typical for TRPC channels. In all three cases, current activation appeared transient, while in Ca^{2+} experiments, the responses appeared sustained (Figure 1 and 2); the reason for this difference is not known. The only difference between the current responses to carbachol and the inhibitors is that carbachol activated a significantly larger current, and acted almost immediately upon addition to the cells, while a variable delay occurred between application of the PI4-K inhibitors and the increase in current. This is consistent with the observations from the Ca^{2+} measurements. The mean, background-subtracted inward currents (at -100 mV) were (mean pA/pF $\pm$ SEM, n): carbachol, -39.6 ± 5.9 (8); LY2942002, -9.3 ± 4.5 (4); wortmannin, -18.5 ± 7.4 (3). The outward currents (at $+100$ mV) were (pA/pF, n): carbachol, 21.4 ± 3.8 (8); LY2942002, 6.4 ± 2.4 (4); wortmannin, 13.4 ± 2.2 (3).

While the perforated patch technique permits current measurement under the most physiological condition, i.e., without dialysis of the cytoplasm, the whole-cell mode has the advantage of permitting introduction of reagents, such as enzymes, antibodies, and small molecules. As shown in Figure 4A, stimulation of TRPC5-expressing HEK293 cells with the muscarinic agonist methacholine activates both small inward (at -100 mV) and larger outward (at $+100$ mV) currents that were absent in wild type HEK293 cells (data not shown). The current-voltage (IV) relationship for TRPC5 current depicted in Figure 4B displayed the characteristic double rectification with strong outward rectification, as previously described for TRPC5 [30,56]. However, the relative magnitude of the inward current is notably smaller than that seen with perforated patch. We also found that responsiveness to muscarinic agonists appeared to wash out after a period of 5–10 minutes, after which time cells seldom responded to receptor activation.

Stimulation of TRPC5-expressing HEK293 cells with $20 \mu\text{M}$ wortmannin or LY2942002 produced inconsistent results in whole-cell experiments. In some cells, wortmannin induced an increase in both inward and outward currents, although the magnitude of the currents was somewhat smaller than that obtained with methacholine (Figure 4C). Wortmannin never induced similar currents in TRPC3-expressing HEK293 cells or HEK293 wild type cells (data not shown). The IV curve obtained after addition of wortmannin to TRPC5-expressing HEK293 cells (Figure 4D) was similar to that obtained following TRPC5 activation through muscarinic agonist stimulation. The inconsistency and tendency to washout of the responses in whole-cell configuration were disappointing because this precluded experiments requiring long dialysis times, for example with antibodies directed against PIP_2 .

Wortmannin-activated TRPC5 currents are inhibited by PIP and PIP_2

Our data so far suggest an inhibitory effect of polyphosphoinositides on TRPC5 channels and that this inhibition is relieved by pharmacological procedures that deplete cellular polyphosphoinositides. We then sought to explore the ability of polyphosphoinositides to inhibit wortmannin-induced TRPC5 currents in HEK293 cells stably expressing TRPC5 cation channels. Unfortunately, as discussed above, effects of these drugs on membrane currents in

the whole-cell configuration, which could be used for introduction of phospholipids, were inconsistent. In a series of experiments in which a 30 min pretreatment with wortmannin induced a consistent increase in whole cell current (an approximate doubling of inward and outward current), cells were then dialyzed with a patch pipette containing either the pipette solution alone (described in the methods section) or the pipette solution containing 20 μM of different polyphosphoinositides. As shown in Figure 5, following break-in, dialysis of cells with PIP_2 (Figure 5A) or PIP (Figure 5B) inhibited wortmannin-activated TRPC5 currents while 20 μM of PIP_3 (Figure 5C) had no effect on TRPC5 currents. The inhibitory effects of polyphosphoinositides occurred after a delay of a minute or so, perhaps due to slow diffusion of the lipids through the patch pipette openings or in the cytoplasm.

Effects of PIP_2 on single TRPC5 channels

We next turned to measurement of the activity of TRPC5 channels in excised patches. This preparation should give rapid access to the intracellular surface of the plasma membrane and the channels in order to investigate their regulation. In excised patches, there was little apparent channel activity in control cells, or in cells that had been pre-treated with PI4-K inhibitors. Addition of PIP_2 to the patches resulted in robust activation of channels with a conductance at +60mV of 64.6 ± 0.1 pS (Figure 6), a value similar to that previously reported for TRPC5 [30]. The channels were also activated albeit to a somewhat lesser extent by PIP_3 . Figure 7 summarizes a series of experiments showing that the channels were significantly activated by PIP_2 , but not by wortmannin, or by the de-acylated PIP_2 , glycerol-phosphoryl-inositol 4,5-bisphosphate (GPIP₂). The channels could also be activated by addition of ATP, possibly indicating that the phosphatidylinositol or PIP precursor and necessary kinases were present in the patches. In one series of experiments (Figure 7C) in which there was measurable spontaneous activity in the patches, this activity was decreased by the PIP_2 scavenger, polylysine (PL).

Effect of membrane targeted PIP_2 5-phosphatase

The results to this point present a paradox: pharmacological and whole-cell patch-clamp data suggest that PIP_2 exerts an inhibitory control on TRPC5, while the single channel data suggests the PIP_2 activates the channels. We consider that the PI4-K inhibitors might affect different pools of PIP_2 than those involved in positive modulation of the channels. In addition, pharmacological inhibition of PI4-K not only decreases the levels of PIP_2 but also depletes, and likely to a greater extent, the levels of PI4P . Therefore, we sought to use a molecular tool to specifically deplete PIP_2 levels and assess its effect on TRPC5 activity. We adopted a strategy recently developed by Varnai et al [35] whereby a cytoplasmic form of the PIP_2 5-phosphatase is targeted to the plasma membrane by rapamycin, as described in the methods section. We confirmed that addition of rapamycin to agonist-activated wild-type cells resulted in rapid cessation of Ca^{2+} signaling, as originally shown by Varnai *et al.* [35]; addition of rapamycin to wild-type cells, or cells transfected with either of the two constructs alone had no such effect (data not shown). Surprisingly, unlike the case for 4-kinase inhibitors, addition of rapamycin to TRPC5-expressing HEK293 cells did not activate TRPC5-mediated Ca^{2+} entry nor did rapamycin potentiate TRPC5-activation in response to low doses of agonist (5 μM) (not shown). Rather, agonist-activated TRPC5-mediated Ca^{2+} entry was strongly inhibited upon addition of rapamycin (Figure 8).

The 4-kinase inhibitors would decrease levels of both PIP and PIP_2 , while the targeted 5-phosphatase would decrease only PIP_2 , possibly increasing PIP. We thus considered the possibility that PIP might act to inhibit TRPC5, antagonizing the activation by PIP_2 . For these experiments, we determined the sensitivity of TRPC5 channels to PIP_2 (Figure 9A), and selected 1 μM PIP_2 as a submaximal concentration. Addition of a large excess of PIP (10 μM) to excised patches from TRPC5-expressing cells together with 1 μM PIP_2 caused no significant

inhibition of TRPC5 channel activity (Figure 9B, C). 10 μ M PIP, when applied alone, caused only modest activation of TRPC5 channel activity as compared to PIP₂ (Figure 9C).

Discussion

The mechanism of activation *via* PLC-coupled receptor stimulation of TRPC5 and TRPC4 has remained one of the unsolved mysteries in the TRPC field [57]. TRPC5 activation was shown to depend upon PLC, but clearly did not involve DAG or IP₃ [30]. Plant and Schaefer reported that receptor-mediated activation of currents in cells transfected with TRPC4 and TRPC5 was unaffected by infusion of low molecular weight heparin [57] ruling out the involvement of IP₃ receptors in TRPC5 activation. Venkatachalam et al. [27] showed that TRPC5 could be activated *via* a muscarinic receptor in DT40 B cells lacking all three IP₃ receptor subtypes. Strübing et al. [58] showed that like homomeric TRPC4 and TRPC5, heteromers formed between TRPC1 and TRPC5 were also not activated by infusion of IP₃, but responded to subsequent muscarinic receptor activation (but see [59]). Otsuguro *et al.* [31] provided the first clues to the possible activation mechanism, demonstrating that TRPC4 activity was inhibited by polyphosphoinositides. Other findings in their study suggested that depletion of polyphosphoinositides played a role in activating TRPC4, but in conjunction with other signals such as G β /G α or a rise in [Ca²⁺]_i [31].

In this study, we show that drugs that are expected to deplete polyphosphoinositides activate TRPC5 cation channels expressed in HEK293 cells, consistent with the Otsuguro *et al.* results, and providing a possible mechanism of activation of TRPC5 channels through PLC-coupled receptor stimulation. We confirmed that the synthetic DAG, OAG, was incapable of activating TRPC5 channels in our TRPC5-expressing HEK293 cell line. However, pharmacological inhibition of PI4-K by wortmannin and LY294002 which causes polyphosphoinositide depletion was able to activate calcium entry and whole-cell TRPC5 current in TRPC5-expressing HEK293 cells with no effect observed in wild type HEK293 cells. We found no evidence for a role for cytoplasmic Ca²⁺ signaling, since the muscarinic agonist as well as the 4-kinase inhibitors activated TRPC5 when cytoplasmic Ca²⁺ was strongly buffered in the whole-cell configuration. Importantly, the 4-kinase inhibitors were unable to activate calcium entry and ionic currents in an HEK293 cell line stably expressing TRPC3 channels. Rather, muscarinic receptor activation of TRPC3 cation channels was completely abrogated by pre-treatment with these drugs. Finally, Activation of TRPC5 currents by wortmannin could be partially reversed by including PIP or PIP₂ but not PIP₃ in the patch pipette. We cannot relate the concentrations of these lipids in the patch pipette to physiological concentrations in the plasma membrane, nor can we determine if either of these lipids can be interconverted to the other in this experimental situation. Thus, we cannot definitively determine which (or if both) of these lipids is the critical regulator of TRPC5. Nonetheless, these findings are consistent with the proposal that receptor-mediated activation of PLC activates TRPC5 through relief of inhibition by polyphosphoinositides.

It is well known that polyphosphoinositides are key regulators of many ion channels and transporters (for reviews see [60,61,62,18]). In the majority of instances, this regulation is thought to be allosteric rather than a primary means of channel activation. Also, in the majority of instances, polyphosphoinositides either activate, or are required for activation of the channels. For most TRP superfamily members, polyphosphoinositides exert positive modulatory effects on channel activity [63,64,61], and we have shown that this is the case for one member of the TRPC family, TRPC7 [65]. Surprisingly, we found that TRPC5 channels in excised patches showed low activity until PIP₂ was added to them. PIP was only a weak activator, and did not appreciably affect the ability of PIP₂ to activate the channels. Also, global depletion of PIP₂ by a membrane-targeted PIP₂ 5-phosphatase strongly inhibited TRPC5 activity. This may mean that the 4-kinase inhibitors target a specific pool of PIP₂, or that

components of the regulatory mechanism for TRPC5 channels is lost in the excised patch. Regardless, it seems clear that in addition to its role in the signaling pathway for TRPC5 activation, PIP₂ also plays a more fundamental positive role in TRPC5 function, similar to that shown for the majority of TRP channels that have been studied. We propose a somewhat speculative model to accommodate these two apparently opposing effects of PIP₂. We suggest that PIP₂ may be intimately associated with TRPC5 channels where it is needed for proper channel function. In addition, we propose an additional role for polyphosphoinositides (PIP₂ or PIP) in a signaling mechanism, perhaps involving a negative regulator that brakes TRPC5 in a polyphosphoinositide-dependent manner. In an excised patch, this regulator may be gone, as well as the PIP₂ necessary for channel activity. Addition of PIP₂ in this circumstance would lead to channel activation. This model is summarized in Figure 10. Note that this model implies spatially restricted pools of polyphosphoinositides fulfilling these distinct functions; consistent with this idea, unlike its precursor, PIP, PIP₂ diffusion in the plasma membrane appears to be highly restricted due to interactions with the cytoskeleton [66]. In a recent study, Lukacs *et al.* [67] presented evidence that PIP₂ can dually regulate TRPV1 channels, depending on the strength of the channel stimulus.

In addition to activation by PLC-coupled receptor agonists, TRPC5 channels have been shown to be activated by store depletion [56], by augmented membrane trafficking [68], and can be activated in a direct manner by high concentrations of lanthanides [38]. Lanthanides are believed to bind to a specific site in the extracellular domain of the channel leading to TRPC5 activation. However, because of the apparent complex roles of polyphosphoinositides, additional work will be needed to identify the exact site(s) on TRPC5 channels involved in phospholipid regulation. Members of the TRPC family of cation channels are increasingly recognized as important players in native calcium entry pathways controlling a wide variety of physiological functions in many cell types. Further work is needed to better understand the regulation of TRPC channels under physiological conditions and how this regulation participates in shaping complex physiological responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Modes of activation of TRPC5

A. HEK293 cells stably expressing TRPC5 channels exhibit a receptor-induced Ca²⁺ entry that is insensitive to low concentrations of Gd³⁺ (5 μM Gd³⁺) that block the endogenous capacitative calcium entry (solid trace). Agonist-induced Ca²⁺ entry in wild type HEK293 (HEK-Wt) cells is completely abolished by 5 μM Gd³⁺ (dotted trace). B. TRPC5 channels can be activated by high concentrations of Gd³⁺ (50 μM). 50 μM Gd³⁺ induces a calcium entry in TRPC5-expressing HEK293 cells (solid trace) but not in wild type cells (dotted trace). C. Unlike TRPC3 channels that can be activated by diacylglycerol analogs (OAG, solid trace), TRPC5-expressing HEK293 cells show no calcium entry after addition of 100 μM OAG (dotted trace). OAG failed to activate TRPC5 even in the presence of protein kinase C inhibitors (data not shown). In A and C, experiments were initiated in the absence of Ca²⁺, and Ca²⁺ restored as indicated, while in B, Ca²⁺ was present throughout. Shown are average traces from 23–58 cells, representative of a total of at least 4 independent experiments.

Figure 2. Phosphatidylinositol 4-kinase (PI-4K) inhibitors activate TRPC5 but not TRPC3

A. Wortmannin (20 μM) induces Ca²⁺ entry in TRPC5-expressing HEK293 cells (solid trace) but has no effect on wild type cells (dotted trace, WT). Gray trace: DMSO control for TRPC5 cells. B. Similar results are obtained for another PI-4K inhibitor, LY294002 (100 μM). C. LY294002 (100 μM) activated a robust Ca²⁺ entry in TRPC5-expressing HEK293 cells (solid trace) while it had no effect on TRPC3-expressing cells (dotted trace). Gray trace, DMSO control for TRPC5 cells. D. LY294002 (100 μM) inhibited TRPC3-mediated Ca²⁺ entry in response to agonist stimulation (methacholine, MeCh 300 μM). Black trace, absence of LY294002; gray trace, presence of LY294002; Iono., 10 μM ionomycin. Shown are average traces from at least 50 cells, representative of a total of 3–8 independent experiments.

Figure 3. The muscarinic agonist carbachol (200 μ M), and the PI4-K inhibitors, LY294002 (100 μ M) and Wortmannin (20 μ M) activate inward and outward currents measured by the perforated patch technique in TRPC5-expressing HEK293 cells

The upper panels show representative time courses of current development (at -100 and $+100$ mV) using the Nystatin-perforated patch mode before and after stimulation with CCh (200 μ M; A), LY294002 (100 μ M, B) and Wortmannin (20 μ M, C). Lower panels show the corresponding current/voltage (I/V) relationship for CCh (D), LY294002 (E) and Wortmannin (F). Sweeps were taken before (a) and after (b) addition of stimuli at the times indicated in the corresponding time course traces (upper panels). In these experiments, series resistance was in the range of 33 – 50 M Ω .

Figure 4. The muscarinic agonist methacholine (300 μ M) and Wortmannin (20 μ M) activate an inward and outward current measured by the whole-cell patch-clamp technique in TRPC5-expressing HEK293 cells

A. Whole cell current densities in a TRPC5-expressing cell sampled at +100 mV and -100 mV. B. Current Voltage (I/V) relationship in a TRPC5-expressing cell subjected to 250 ms voltage ramps between -100 and +100 mV, before (black trace) and after (gray trace) addition of methacholine. Representative of 6 independent experiments. C. Wortmannin (20 μ M) induces an increase in both inward and outward whole cell currents in TRPC5-expressing cells, with no effect on HEK-Wt or TRPC3-expressing HEK293 cells (not shown). The data are averages of the maximum inward and outward currents before and after wortmannin addition from 17 (control) and 15 (wortmannin) experiments. D. The IV curve obtained after addition of wortmannin (20 μ M) to TRPC5-expressing cells is similar to that obtained after stimulation of these cells with methacholine (see B). Representative data from a single experiment from the experiments summarized in C.

Figure 5. PIP₂ and PIP, but not PIP₃ inhibit wortmannin-activated TRPC5 currents

A. TRPC5-expressing HEK293 cells were treated with wortmannin (20 μ M) for 30 minutes. Whole cell measurements were made in the absence (open circles, $n=6$) or presence (filled circles) of 20 μ M PIP₂ ($n=6$, panel A), 20 μ M PIP ($n=5$, panel B), or 20 μ M PIP₃ ($n=6$, panel C) in the patch pipette. After break-in, there was a brief period of current run-up (~ 10 pA/pF). Following stabilization of the current, (~ 50 sec), data (outward at +100 mV, inward at -100 mV) were collected and normalized by ratio to the initial current density (I_0). The differences (control vs. lipid-treated) in inward and outward currents at the end of data collection were analyzed by t-tests; the levels of significance were: PIP₂, inward, $P = 0.059$ (NS), outward, $P = 0.004$; PIP, inward, $P = 0.049$, outward, $P = 0.012$; PIP₃, inward, $P = 0.445$ (NS), outward, $P = 0.519$ (NS).

Figure 6. PIP₂ directly activates TRPC5 channels in the inside-out configuration

A: Representative traces showing TRPC5 activity at +60 mV immediately after excising a membrane patch from a cell exposed to wortmannin (top trace), and after a subsequent application of 10 μ M PIP₂ (bottom trace). B: Corresponding NPo versus time plots for the same cell. Arrows indicate solution exchanges.

Figure 7. TRPC5 is specifically activated by PIP₂ at the single channel level

A: Histograms summarizing changes observed in NPo at +60 mV during inside-out experiments where patches were exposed to a regular external solution (cont.), and then to the same solution supplemented with 20 μ M wortmannin alone (WT) or in the presence of 10 μ M PIP₂; representative of 4 patches obtained in 4 independent cells. B: Same as in A, but patches were exposed to 5 mM ATP alone (ATP) or in the presence of 10 μ M PIP₂ (ATP+PIP₂); representative of 4 patches obtained in 4 independent cells. C: Same as A and B, but patches were sequentially exposed to the regular external solution (Cont.), 10 μ M GPIIP₂, 0.5 μ g/ml poly-L-Lysine (PL) and 10 μ M PIP₂; representative of 6 patches obtained in 6 independent cells.

Figure 8. PIP2 depletion using plasma membrane targeted- PI5-phosphatase inhibits TRPC5
Time course of average fluorescence ratio in TRPC5-expressing HEK293 cells loaded with Fura-2. Cells were exposed to a maximal dose of Carbachol (CCh: 100 μM) in the presence of 2 mM external Ca^{2+} solution and a low dose of Gd^{3+} (5 μM) to block the endogenous store-operated Ca^{2+} entry pathway, as indicated by the horizontal arrow bars. Once the TRPC5-mediated Ca^{2+} entry fully developed, rapamycin (100 nM) was added where indicated. The trace shown represents average data of 21 cells from a recording representative of at least four separate experiments.

Figure 9. PIP weakly activates TRPC5 channels, but does not interfere with the ability of PIP_2 to activate the channels

A: Concentration-response curve showing the concentration-dependent effects of bath applied PIP_2 on TRPC5 channels. Single channel events were recorded at 80 mV ($10 \mu\text{M}$: $n=4$; $1 \mu\text{M}$: $n=10$; 500 nM : $n=4$; 100 nM : $n=4$). **B:** Representative traces showing TRPC5 activity before, during and after the simultaneous addition of $1 \mu\text{M} \text{PIP}_2$ and $10 \mu\text{M} \text{PIP}$ to the cytosolic side of the membrane patch through the bathing solution. Arrow on top trace indicates where the 300 msec bottom trace was taken, showing TRPC5 activity under the presence of both PIP and PIP_2 . **C:** Bar graph indicating the effects of $1 \mu\text{M} \text{PIP}_2$ alone ($n=10$), $10 \mu\text{M} \text{PIP}$ alone ($n=3$), or PIP_2 plus PIP ($n=7$) (added simultaneously as seen in panel B), as well as TRPC5 basal activity recorded before the addition of PIP or PIP_2 ($n=8$). Error bars indicate means $\pm$ SEM.

Figure 10. Model for PIP₂ regulation of TRPC5 channels

In intact cells, agonist (Ag) activation of PLC, or inhibition of PI4-K by wortmannin, leads to reduction of PIP or PIP₂, and dissociation of a polyphosphoinositide-dependent inhibitor of TRPC5. PIP₂ associated with the channel is required for activity; thus, removal of the PIP₂-dependent inhibitor leads to channel activation. In excised patches, both the PIP₂ required for channel activity, as well as the inhibitor are lost; thus addition of PIP₂ leads to robust channel activation.