Phosphatidylinositol 4,5-Bisphosphate Rescues TRPM4 Channels from Desensitization

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TRPM4 is a Ca\(^{2+}\)-activated nonselective cation channel that regulates membrane potential in response to intracellular Ca\(^{2+}\) signaling. In lymphocytes it plays an essential role in shaping the pattern of intracellular Ca\(^{2+}\) oscillations that lead to cytokine secretion. To better understand its role in this and other physiological processes, we investigated mechanisms by which TRPM4 is regulated. TRPM4 was expressed in CHO-K1 cells, and currents were measured in excised patches. Under these conditions, TRPM4 currents were activated by micromolar concentrations of cytoplasmic Ca\(^{2+}\) and progressively desensitized. Here we show that desensitization can be explained by a loss of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P\(_2\)) from the channels. Poly-l-lysine, a PI(4,5)P\(_2\) scavenger, caused rapid desensitization, whereas MgATP, at concentrations that activate lipid kinases, promoted recovery of TRPM4 currents. Application of exogenous PI(4,5)P\(_2\) to the intracellular surface of the patch restored the properties of TRPM4 currents. Our results suggest that PI(4,5)P\(_2\) acts to uncouple channel opening from changes in the transmembrane potential, allowing current activation at physiological voltages. These data argue that hydrolysis of PI(4,5)P\(_2\) underlies desensitization of TRPM4 and support the idea that PI(4,5)P\(_2\) is a general regulator for the gating of TRPM ion channels.

The transient receptor potential (TRP)\(^2\) family of ion channels contains 31 mammalian members, many of which are activated downstream of second messenger signaling pathways (1, 2). TRPM4, a member of the melastatin-related branch of the TRP channels, is a monovalent selective ion channel that is activated by cytoplasmic Ca\(^{2+}\) (3). It is most closely related to TRPM5, which also forms a Ca\(^{2+}\)-activated cation channel and is involved in some forms of taste transduction (4–8). TRPM4 is widely expressed, and its primary role appears to be to regulate membrane potential in response to intracellular Ca\(^{2+}\) signaling (3). In lymphocytes it is an essential component of the feedback network that generates intracellular Ca\(^{2+}\) oscillations that lead to cytokine secretion (9). It also has been proposed to play a role in myogenic constriction of cerebral arteries (10). Both TRPM4 and TRPM5 appear to be activated downstream of phospholipase C (PLC) signaling, most likely through the direct effects of store-released Ca\(^{2+}\) on channel gating (3, 6–8).

The membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PI(4,5)P\(_2\)) has emerged as an important regulator of many ion channels and transporters (11, 12). PI(4,5)P\(_2\) serves as the substrate for PLC, which generates the second messengers inositol 1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol. However, in an increasing number of cases it has become apparent that a decrease in PI(4,5)P\(_2\), and not the increase in IP\(_3\) or diacylglycerol, is the physiologically relevant signal. For instance, KCNQ2/3 channels, which comprise the M current, close in response to the decrease in resting PI(4,5)P\(_2\) levels following activation of PLC by G\(_\alpha\)-linked membrane receptors (13, 14). PI(4,5)P\(_2\) has also been shown to be an important regulator of several TRP channels. TRPV1 becomes hypersensitive to heat and capsaicin following hydrolysis of PI(4,5)P\(_2\), and this underlies the regulation of TRPV1 by inflammatory neurochemicals such as bradykinin (15, 16). Breakdown of PI(4,5)P\(_2\) causes TRPM7 channels to close (17), and regeneration of PI(4,5)P\(_2\) is necessary for sustained gating of Drosophila TRP channels in photoreceptors in response to light (18). Breakdown of PI(4,5)P\(_2\) also appears to underlie desensitization of several TRP channels including TRPM5, TRPM8, and TRPV1 (6, 19–21).

The sensitivity of TRPM4 channels to Ca\(^{2+}\) changes subsequent to activation (22), which may serve a physiologically important role in allowing the channels to respond over a greater stimulus range. In previous work, we found that TRPM5 channels also desensitize to Ca\(^{2+}\) and that sensitivity could be recovered by exogenous PI(4,5)P\(_2\) (6). Similarly it was shown recently that PI(4,5)P\(_2\) restores sensitivity of TRPM8 channels to cold and menthol following desensitization (20, 21). PI(4,5)P\(_2\) regulation of TRPM4 thus seems likely. Here we show that TRPM4 currents exhibit pronounced rundown following initial exposure to Ca\(^{2+}\) and that exposure to PI(4,5)P\(_2\) leads to full recovery of the currents. Moreover we find that manipulations that are expected to change endogenous PI(4,5)P\(_2\) levels predictably enhance or diminish desensitization of TRPM4. Together these data indicate that hydrolysis of PI(4,5)P\(_2\) underlies desensitization of TRPM4.

**MATERIALS AND METHODS**

**Constructs and Expression in Heterologous Cells**—For most experiments we used TRPM4b fused to the C terminus of cyan fluorescent protein under a cytomegalovirus promoter (eGFP-C2 vector, Clontech) as described previously (23). For experiments in Fig. 2, D, E, and F, we used human TRPM4b (hTRPM4b) fused to the C terminus of enhanced green fluorescent protein in pcDNA3.1 (Invitrogen) as described previously (23). CHO-K1 cells were plated on glass coverslips and transfected using FuGENE (Roche Applied Science) or Effectene (Qiagen) accord-


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ing to the manufacturers’ instructions. Recordings were performed 48–72 h after transfection at room temperature.

*Electrophysiology*—ChoK1 cells on coverslips were transferred to the recording chamber, and cells expressing CFP-mTRPM4 were identified under epifluorescence. After formation of a gigaohm seal, the patch was excised into zero Ca²⁺-containing solution (see below) and placed in front of a linear array of microperfusion pipes from which test solutions were delivered (Warner Instruments). For all experiments, the membrane potential was held at −80 mV.

The pipette solution contained 150 mM NaCl, 10 mM HEPES, 2 mM CaCl₂ (pH 7.4 with NaOH). Bath (cytosolic) solutions were as follows. Zero Ca²⁺ solution was 150 mM NaCl, 10 mM HEPES, 2 mM HEDTA (pH 7.4 with NaOH). Solutions with micromolar concentrations of free Ca²⁺ were obtained by adding Ca²⁺ to this HEDTA-based solution as follows. 1.9 mM CaCl₂ was added for 50 µM free Ca²⁺. Solutions containing 100 µM to 5 mM Ca²⁺ were made by adding the appropriate concentration of CaCl₂ without buffer to a solution containing 150 mM NaCl, 10 mM HEPES (pH 7.4 with NaOH). All Ca²⁺ concentrations are reported as calculated with MaxChelator (www.stanford.edu/~cpatton/maxc.html) and were confirmed by direct measurement with a Ca²⁺-sensitive electrode (Microelectrodes, Inc., Bedford, NH). Biochemicals used were as follows: dC₆P₁(4,5)P₂, dC₄P₁(3,4)P₂, dC₆P₁(3,5)P₂, dC₄P₁(3,4,5)P₃, dC₆P₁(4)P (Echelon Bioscience, Salt Lake City, UT), MgATP, Na₂ATP, poly-L-lysine (4–15 kDa), MgGTP, MgADP, arachidonic acid, LY-294002 hydrochloride (Sigma), 1,2-dioctanoyl-sn-glycerol (Avanti Polar Lipids, Alabaster, AL), and wortmannin (Calbiochem).

All recordings were made with an Axopatch 200B amplifier, digitized with a Digidata 1322a, acquired with pClamp 8.2, and analyzed with Clampfit 8.2 (Axon Instruments, Union City, CA). Recordings were sampled at 5 kHz and filtered at 1 kHz. Representative data shown in figures were further decimated 10-fold before exporting into the graphics programs Origin (Microcal, Northampton, MA) and Corel draw (Corel Corp.). Patch pipettes (1.5–3 megaohms) were fabricated from borosilicate glass.

*Data Analysis*—All data were analyzed by Clampfit (Axon Instruments), and curve fitting and statistical analyses were done in Origin (Microcal). Dose-response curves were fitted according to Equation 1,

\[
y = \frac{y_{\text{max}}}{EC_{50}^n} + \frac{[x]^n}{EC_{50}^n} \quad (\text{Eq. 1})
\]

where EC₅₀ is the concentration for half-maximal effect, [x] is the concentration of the substance being tested, and n is the Hill coefficient.

Determination of conductance as a function of voltage (g(V)) was obtained by measuring isoionic tail currents (4 ms after the return to the test potential of −120 mV), normalizing the data to the peak current and fitting it with the Boltzmann equation (Equation 2),

\[
g = \frac{g_{\text{max}}}{1 + e^{-\frac{V_m - V_{1/2}}{k}}} \quad (\text{Eq. 2})
\]

where Vₘₚ is the membrane potential, Vₜ/₂ is the membrane potential at which the current is half-activated, and f is the fraction of current that is not voltage-sensitive. Data were obtained from excised patches where series resistance was measured to be 4–6 megaohms, and series resistance errors (uncorrected) were less than 25 mV at a test potential of +160 mV.

Time constants for deactivation of TRPM4 currents were obtained by fitting the data to Equation 3,

\[
I = I_{\infty} + Ae^{-(t_1)} \quad (\text{Eq. 3})
\]

where I is the current amplitude, Iₙ is the current amplitude at time = 0, t is time, and τ is the time constant. The fraction of current that was voltage-insensitive was calculated as I/(_∞ + A).

All data are presented as mean ± S.E. In some cases error was small, and error bars are within the symbols. Statistical significance was tested by two-tailed Student’s t test.

**RESULTS**

*Recovery of TRPM4 Currents from Rundown by MgATP*—To study second messenger regulation of TRPM4 channels, we expressed mouse TRPM4 fused to cyan fluorescent protein in ChoK1 cells, which otherwise show little endogenous TRPM4-like channel activity. Consistent with previous reports (e.g., Ref. 24), in excised inside-out patches, TRPM4 currents could be evoked in response to 100 µM cytosolic Ca²⁺, and this Ca²⁺-evoked current ran down over several seconds (Fig. 1A).

We will show later that this rundown is associated with a change in Ca²⁺ sensitivity of the channels, and thus can be formally considered desensitization. The two terms are therefore used interchangeably here. Although the rate of rundown was variable, overall it appeared to be faster at higher cytoplasmic Ca²⁺ concentrations (data not shown) as observed for TRPM5 (6). We also measured TRPM4b currents in whole cell recording from ChoK1 cells. Upon intracellular dialysis of 50 µM Ca²⁺, large outwardly rectifying currents were activated that decayed at a rate similar to the currents evoked in excised patch recording (see supplemental Fig. 1). All remaining experiments were done in excised patch mode where the intracellular milieu could be rapidly and reproducibly controlled.

Previous work has shown that in excised patch recording mode addition of MgATP to the cytoplasmic/bath solution leads to recovery of TRPM4 currents from rundown (22). Likewise we found that a 40-s exposure of the cytosolic surface of the patch to a Ca²⁺-free solution containing 2 mM MgATP and 2 mM free ATP produced complete recovery of TRPM4 currents that had been evoked in response to 100 µM cytosolic Ca²⁺ (Fig. 1A and B). Free adenine nucleotides, including ATP, block TRPM4 and native TRPM4-like channels (25, 26), and thus it is possible that the recovery of the currents is related to this blocking effect of ATP. However, free ATP did not promote recovery of TRPM4 currents indicating that these processes are not linked (22) (Fig. 1A and B). Moreover the effect of MgATP lasted tens of seconds after its washout, making it unlikely that this effect was due to direct binding of MgATP to the channel (22). MgADP and MgGTP were ineffectual at restoring the TRPM4 current, indicating that this effect is not due to Mg²⁺ itself (Fig. 1B). Together these data are consistent with the involvement of a kinase in restoration of the current.

Cytosolic MgATP has been demonstrated to induce recovery of Pl(4,5)P₂-sensitive ion channels from rundown (11, 12, 27) most likely by activating phosphatidylinositol phosphate kinases, thereby promoting regeneration of Pl(4,5)P₂ (28, 29). For example, Kir6.2 currents can be recovered by MgATP, and this effect is blocked by the lipid kinase inhibitor wortmannin (27, 30). To determined whether the ability of MgATP to restore TRPM4 currents could be attributed to activation of a lipid kinase, we tested whether wortmannin, at concentrations that block phosphatidylinositol (Pi) 4-kinase (PI4K) and PI 3-kinase (PI3K) (31), interfered with MgATP-induced recovery of TRPM4. Preincubation of cells for 5 min with 50 µM wortmannin significantly attenuated the recovery of TRPM4 currents by MgATP (Fig. 1C) supporting a role for PI4K or PI3K in this process. In contrast, we found no effect of the PI3K-specific blocker LY294002 (32) (100 µM for 1 h) on recovery of
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TRPM4 currents by MgATP (Fig. 1C), suggesting that this process is not mediated by PI3K.

PI(4,5)P₂ Recovers TRPM4 Currents from Rundown—These data suggest that the recovery of the TRPM4 current by MgATP might be attributed to an increase in PI(4,5)P₂ in the membrane and conversely that the initial rundown of TRPM4 currents might be attributed to a loss of PI(4,5)P₂ from the patch. According to this hypothesis, manipulations that deplete PI(4,5)P₂ should accelerate decay of the TRPM4 currents. Poly-i-lysine is a PI(4,5)P₂ scavenger, and it has been widely used to demonstrate regulation of channels and transporters by endogenous PI(4,5)P₂ (11, 12). In excised patches where TRPM4 currents showed slow rates of rundown, exposure to poly-i-lysine accelerated rundown, supporting a role for PI(4,5)P₂ in regulating TRPM4 channel activity (Fig. 2A).

If hydrolysis of PI(4,5)P₂ underlies rundown of TRPM4 currents, then application of exogenous PI(4,5)P₂ should lead to recovery of the currents. To test this, we waited until TRPM4 currents evoked in response to 100 μM cytoplasmic Ca²⁺ (V_m = −80 mV). A, a patch from a ChoK1 cell expressing mTRPM4 shows slow rundown, which is accelerated by exposure to the PI(4,5)P₂ scavenger poly-i-lysine (PLL; 30 μM) (13), in a patch with fast rundown, diC₈-PI(4,5)P₂ (10 μM) restores the mTRPM4 current to its initial magnitude. C, average data showing the initial magnitude of the mTRPM4 currents, the magnitude of the currents after rundown, and the magnitude of the currents after recovery by PI(4,5)P₂ (10 μM) measured using a protocol similar to that in B (n = 29). D, rundown of hTRPM4 expressed in ChoK1 cells is enhanced by poly-i-lysine (PLL; 10 μM). E, 10 μM PI(4,5)P₂ recovers the hTRPM4 current from rundown. F, average data showing the magnitude of the hTRPM4 currents initially, after poly-i-lysine, and after exposure to PI(4,5)P₂ (10 μM) (n = 5). G and H, a patch in which TRPM4 currents decayed very little shows a small response to PI(4,5)P₂ (G), whereas a patch in which TRPM4 currents have run down substantially shows a large response to PI(4,5)P₂ (H). I, the fold rundown, measured as the peak initial current divided by the peak current in response to PI(4,5)P₂. Data from the two patches shown in G and H are circled. A linear fit to the data is shown (slope = 0.67 ± 0.02). The degree of correlation between these two measurements (r = 0.86, p < 0.001) suggests that PI(4,5)P₂ acts to restore the current following rundown. PLL, phosphatidyllysine bisphosphate.
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FIGURE 3. Specificity and sensitivity of TRPM4 currents for PI(4,5)P$_2$. A, TRPM4 currents were evoked in inside-out patches (V$_m$ $= -80$ mV) by exposure to 100 nM cytoplasmic Ca$^{2+}$. A, TRPM4 current is recovered following rundown by PI(4,5)P$_2$, but not by 1,2-dioctanoyl-sn-glycerol (DAG) or arachidonic acid (AA). B, summary of experiments testing the ability of a range of second messengers and phosphatidylinositols to recover the TRPM4 current. Data were obtained from experiments as in A and were normalized to the response to PI(4,5)P$_2$, using the equation: relative $I$ = (I/$(I_{Ca^{2+}})-(I_{Ca^{2+}})/(I_{Ca^{2+}}) - (I_{Ca^{2+}})/$) where $I_{Ca^{2+}}$ is the current that was evoked in response to Ca$^{2+}$ after desensitization. All phosphatidylinositols were present at a concentration of 10 $\mu$m, and the responses were normalized to the response to 10 $\mu$m PI(4,5)P$_2$. C and D, average dose-response data for recovery of TRPM4 currents by PI(4,5)P$_2$ is fit with an EC$_{50}$ of 5.8 ± 0.9 and $n_H$ = 2.1 ± 0.5 (number of patches is given above each data point in parentheses). Only patches in which the TRPM4 current decayed nearly completely were used in this analysis. Relative $I$ was calculated as (I/$(I_{Ca^{2+}})-(I_{Ca^{2+}})/(I_{Ca^{2+}}) - (I_{Ca^{2+}})/$) where $I_{Ca^{2+}}$ is the current that was evoked in response to Ca$^{2+}$ after desensitization. PI$_3$P$_2$ phosphatidylinositol bisphosphate.

These data are consistent with the possibility that PI(4,5)P$_2$ is initially bound to the channel (or a subunit of the channel complex) and unbinding of PI(4,5)P$_2$ underlies rundown. If this is the case, then PI(4,5)P$_2$ should modulate the currents most effectively when they have run down. To test whether this is the case, we took advantage of the fact that in some patches, rundown was slow and incomplete. For example, we found that 30 s after exposure to 100 $\mu$M Ca$^{2+}$, the current had run down less than 50% in seven of 26 patches (Fig. 2G), and in three of these patches it had run down less than 2%. Representative data shown in Fig. 2, G and H, show that PI(4,5)P$_2$ produces a more robust enhancement of the current in patches where the current has run down. To quantify this result, for each recording of TRPM4 currents, we measured the fold rundown after Ca$^{2+}$ exposure and the fold enhancement of the current by PI(4,5)P$_2$. As seen in Fig. 2F, we found a direct correlation between these two processes indicating that PI(4,5)P$_2$ is most effective at enhancing the currents when they have undergone rundown ($r = 0.89, p < 0.001, n = 26$).

Specificity and Sensitivity of Recovery of TRPM4 Currents by PI(4,5)P$_2$—The observed effects of PI(4,5)P$_2$ could be due to the products of its hydrolysis. PI(4,5)P$_2$ can be hydrolyzed by PLC into diacylglycerol and IP$_3$ and by phospholipase A$_2$ into arachidonic acid, and all three of these second messengers have been shown to regulate one or more types of TRP channels (34–37). Moreover IP$_3$, along with PI(4,5)P$_2$, binds to some pleckstrin homology domains (38). Thus we tested the ability of each of these second messengers to restore TRPM4 currents following rundown. No restoration of the TRPM4 current was observed in response to application of either arachidonic acid (50 $\mu$M), 1,2-dioctanoyl-sn-glycerol (the diC$_2$ analog of diacylglycerol; 50 $\mu$M), or IP$_3$ (10 $\mu$M) in the same patches in which robust restoration of the current by PI(4,5)P$_2$ was observed (Fig. 3A and B). We conclude that the ability of PI(4,5)P$_2$ to restore TRPM4 currents following rundown is not due to effects of products of its hydrolysis.

To further determine the specificity of regulation of TRPM4 by PI(4,5)P$_2$, we compared the effects of the most abundant singly, doubly, or triply phosphorylated PIs (Fig. 3B). All PIs were tested for their ability to recover TRPM4 currents in excised inside-out patches in response to 100 nM cytoplasmic Ca$^{2+}$ as compared with PI(4,5)P$_2$. The singly phosphorylated PI, PI(4)P, which is as abundant as PI(4,5)P$_2$ (39), was ineffective at regulating TRPM4. The doubly phosphorylated PIs, PI(3,4)P$_2$ and PI(3,5)P$_2$, were equally effective as PI(4,5)P$_2$. The triply phosphorylated PI, PI(3,4,5)P$_3$, although somewhat more effective than PI(4,5)P$_2$, is present at less than 1% of the concentration of PI(4,5)P$_2$ (39). The substrate specificity that we observed for TRPM4 is similar to that observed for Kir6.2 (40), a subunit of the K$_{ATP}$ channel that is regulated by PI(4,5)P$_2$.

To determine sensitivity of TRPM4 currents to PI(4,5)P$_2$, we measured the effects of varying concentrations of PI(4,5)P$_2$ on the recovery of TRPM4 currents in excised inside-out patches evoked in response to 100 nM Ca$^{2+}$. We observed a dose-dependent recovery of the currents that could be fit with an EC$_{50}$ of 4.3 $\mu$M and a Hill coefficient of 2.0 (Fig. 3, D and E). 10 $\mu$M PI(4,5)P$_2$, a concentration that fully restored TRPM4 currents, produced a near saturating effect.

PI(4,5)P$_2$ Restores Ca$^{2+}$ Sensitivity of TRPM4 Currents—To further test whether PI(4,5)P$_2$ was likely to be an endogenous regulator of TRPM4, we determined whether the functional properties of TRPM4 currents after exposure to PI(4,5)P$_2$ mimicked those before rundown. The Ca$^{2+}$ sensitivity of TRPM4 has been reported to change during desensitization; this was because of a change in Ca$^{2+}$ sensitivity. Immediately upon patch excision, large TRPM4 currents could be evoked in response to micromolar concentrations of cytoplasmic Ca$^{2+}$ with an EC$_{50}$ of 110 ± 28 $\mu$M and a Hill coefficient (n$_H$) of 3.7 ± 0.4 (n = 6) (Fig. 4, A and B). To induce full rundown of TRPM4 currents, we exposed the patch for 60 s to 1 mM Ca$^{2+}$

Open Probability Decreases during Desensitization and Is Restored by PI(4,5)P$_2$—We next determined whether PI(4,5)P$_2$ restored the single channel properties of TRPM4 that had changed during desensitization. Changes in TRPM4 currents during desensitization could be due to...
either changes in the gating of the channels or changes in their conductance. To distinguish between these possibilities, we recorded from excised inside-out patches that contained few single channels where unitary currents could be readily resolved (Fig. 5). Prior to desensitization, the single channel current was \( 2.45 \pm 0.04 \text{ pA} \) \((n = 5)\) at \(-80 \text{ mV}\), corresponding to a chord conductance of 30 picosiemens. The magnitude of the single channel currents did not change during desensitization and recovery by PI(4,5)P_2 \((i = 2.45 \pm 0.06 \text{ pA}, n = 6 \text{ and } i = 2.56 \pm 0.04 \text{ pA}, n = 7, \text{ respectively})\); see Fig. 5).

In contrast, we observed a dramatic change in the gating of the channels. This is illustrated in the recording in Fig. 5. This patch was unusual in that only one channel recovered from desensitization following exposure to PI(4,5)P_2, and it afforded us the opportunity to observe the gating of a single modified channel (note that if more than one channel has been modified we would have seen more instances where two channels opened simultaneously; these instances were rare in this patch). By visual inspection we found that channel openings lasted for hundreds of milliseconds, and bursts of openings lasted for many seconds prior to desensitization, whereas after desensitization channels opened for only a few milliseconds. Exposure to PI(4,5)P_2 promoted bursts of openings that lasted many seconds, apparently restoring the initial gating behavior of the channels (Fig. 5).

PI(4,5)P_2 Uncouples Channel Opening from Voltage Sensing of TRPM4 Channels—It has been proposed recently that PI(4,5)P_2 regulates the gating of TRPM8 currents by changing the voltage dependence of activation (21). Like TRPM8, TRPM4 currents show pronounced outward rectification as a result of voltage-dependent gating (24). To determine whether voltage-dependent gating of TRPM4 is modulated by PI(4,5)P_2, we measured TRPM4 currents in excised inside-out patches in response to ramp depolarizations (either 1 or 0.1 V/s) upon first exposure to \( \text{Ca}^{2+} \), during desensitization, and following recovery by PI(4,5)P_2. In the presence of 500 \( \mu \text{M} \) \( \text{Ca}^{2+} \), we found that the rectification of the TRPM4 currents changed significantly during desensitization with little rectification at the peak of the current \((I_{\text{peak}}/I_{\text{rest}} = 2.2 \pm 0.3, n = 5)\) and strong rectification observed following desensitization (Fig. 6, A, B, and C). \( I_{\text{peak}}/I_{\text{rest}} = 5.9 \pm 0.9, n = 10)\). PI(4,5)P_2 completely restored the nearly linear current-voltage relation of the currents prior to desensitization (Fig. 6, A, B, and C; \( I_{\text{peak}}/I_{\text{rest}} = 1.9 \pm 0.3, n = 10)\). We obtained similar results when TRPM4 was activated with 100 \( \mu \text{M} \) cytoplasmic \( \text{Ca}^{2+} \) (data not shown).

The conversion of the current-voltage relation from outwardly rectifying to nearly linear suggests that PI(4,5)P_2 acts to change the voltage dependence of activation of TRPM4 currents. To examine changes in voltage-dependent gating of TRPM4 directly, we measured TRPM4 currents activated by 500 \( \mu \text{M} \) \( \text{Ca}^{2+} \) in response to families of voltages steps before and after exposure of the patch to PI(4,5)P_2 (Fig. 6D). By measuring the magnitude of the current immediately upon repolarization of the membrane potential to \(-120 \text{ mV}\) (isochronal tail currents) as a function of the step potential, we obtained a measure of the conductance as a function of voltage \((g(V))\) (Fig. 6E). \( g(V) \) curves were fit with the Boltzmann equation, which describes the midpoint \((V_{1/2})\) and steepness \((\text{dx})\) of steady-state voltage-dependent activation. This analysis showed that in the desensitized state TRPM4 is weakly dependent on voltage \((\text{dx} = 44.6 \pm 5.8 \text{ mV})\) with a relatively depolarized midpoint of activation \((V_{1/2} = 28.4 \pm 8.8 \text{ mV}, n = 10; \text{Fig. } 6E)\). At negative voltages the conductance approached 16.2 \( \pm \) 2.2% \((n = 10)\) of its initial value, and we interpret this as meaning that \( \sim 16\% \) of the current is not voltage-sensitive. The same voltage protocol was used to measure the voltage dependence of TRPM4 currents after recovery by PI(4,5)P_2 (20 \( \mu \text{M} \)) (Fig. 6, D and E). We found no significant change in the slope of the Boltzmann fit \((\text{dx} = 52.7 \pm 4.9 \text{ mV}, n = 7)\) or in the midpoint of activation \((V_{1/2} = 13.3 \pm 14.6 \text{ mV}, n = 7)\) as compared with the data obtained in the absence of PI(4,5)P_2. Instead we observed a significant increase in the fraction of the current that was not voltage-sensitive \((33.5 \pm 7.5\%, p < 0.05; n = 7, \text{ Fig. } 6D)\). Thus PI(4,5)P_2 affects the
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FIGURE 6. PI(4,5)P₂ changes rectification of TRPM4 currents by releasing channels from voltage-dependent gating. A, time course of currents measured at +160 mV (filled circles) and −160 mV (open circles) during desensitization and recovery by PI(4,5)P₂. Recordings were made in excised inside-out patch mode; membrane potential was −80 mV and was ramped from −160 to +160 mV every second as in E. Cytoplasmic (bath solution) contained 500 μM Ca²⁺. α–d indicate times at which current traces are plotted in B, B, representative current traces taken at the times indicated in A. The stimulus was a ramp depolarization from −160 to +160 mV (1 V/s). C, traces from B scaled to the current at +160 mV. Note that the initial trace and the trace obtained after recovery by PI(4,5)P₂ show little outward rectification, whereas the trace taken after the currents had desensitized shows pronounced outward rectification. D, current families recorded in the presence of 500 μM Ca²⁺ after desensitization and following exposure to phosphatidylinositol bisphosphate (PIP₂). The voltage protocol is shown below the traces. Note that there is a large change in the baseline current and little change in the kinetics of the currents in response to PI(4,5)P₂. Leak current (measured in the absence of Ca²⁺) was less than 5% of the Ca²⁺-induced current (V_m = −100 mV) and was not subtracted. E, conductance as a function of voltage (g(V)) data obtained by measuring isochronal tail currents from families of current traces as those shown in D. Tail currents were normalized to the maximal tail current, and data shown are the average from nine and six patches (desensitized and phosphatidylinositol bisphosphate, respectively). The data were fit with the Boltzmann equation. A large component of current after PI(4,5)P₂ exposure appeared to have no voltage dependence as evident by the fact that the fit to the Boltzmann equation asymptotes at a g/V_m of 0.4. Note that we could not measure the g(V) relation before desensitization due to the fact that currents were not stable during the long time required to obtain the data. F, deactivation kinetics of TRPM4 currents before and after PI(4,5)P₂ exposure in the presence of 500 μM Ca²⁺. Deactivation kinetics were measured at −120 mV following a voltage step to +160 mV (as in D) and were fit with a single exponential time constant (fit shown superimposed on the data). G, average data for the rates of activation and deactivation of TRPM4 in response to voltage steps in the presence or absence of PI(4,5)P₂. Data are from nine and six patches (desensitized and phosphatidylinositol bisphosphate, respectively). Both deactivation and activation slowed in response to PI(4,5)P₂, although only the change in deactivation was significant (t, p < 0.01). Activation of TRPM4 currents elicited in response to a voltage step to +160 mV from −100 mV (as in D), and the time at which the current relaxation due to voltage-dependent gating was 50% complete (t½) was measured.

steady-state voltage dependence of TRPM4 primarily by promoting voltage-independent opening.

To further examine the effect of PI(4,5)P₂ on voltage-dependent gating of TRPM4, we analyzed the kinetics of activation and deactivation of the currents following voltage steps. In the presence of 500 μM Ca²⁺, in response to repolarization of the membrane potential from +160 mV to −120 mV, TRPM4 currents showed a time-dependent relaxation that was well fit by a single exponential time constant (Fig. 6F). After PI(4,5)P₂ exposure, there was a significant slowing in the time constant for decay (τ = 56.5 ± 5.4 ms before PI(4,5)P₂ as compared with τ = 34.6 ± 3.6 ms before; Fig. 6G; p < 0.05) and a significant increase in the sustained fraction of current (I_/I_ = 36.8 ± 7.2% after PI(4,5)P₂ as compared with 17.5 ± 3.0% before; p < 0.05). We also examined the rate of activation of TRPM4 currents in response to depolarization to +160 mV in the presence and absence of PI(4,5)P₂. To quantify the rate of activation, which was not well described by a single exponential time constant, we measured the time to half-activation. There was a tendency for activation, like deactivation, to be slower after PI(4,5)P₂ exposure, although this tendency did not achieve statistical significance (Fig. 6G). Overall these data show that PI(4,5)P₂ significantly increases the fraction of current that is not sensitive to voltage with more subtle effects on activation and deactivation rates of TRPM4.

DISCUSSION

Recently there has been intense interest in the mechanisms by which members of the TRP family of ion channels are regulated. Many of these channels are activated downstream of PLC signaling and thus provide a mechanism for coupling this enzymatic pathway to changes in membrane potential (1, 2). Two TRP channels, TRPM4 and TRPM5, are directly activated by intracellular Ca²⁺ subsequent to its release from intracellular stores (3, 6–8). We now show that a second component of the PLC signaling pathway, PI(4,5)P₂, is a potent co-activator of TRPM4. PI(4,5)P₂ has no effect in the absence of activation by Ca²⁺, and the two signaling molecules act in concert to produce maximal activation of TRPM4. From a theoretical perspective, it seems likely that TRPM4 will be most strongly activated upon initial stimulation of the PLC signaling pathway when Ca²⁺ levels are high and PI(4,5)P₂ levels are still elevated, and its activity will progressively decline as PI(4,5)P₂ levels fall. These features will allow TRPM4 to report changes in PLC signaling and to ignore sustained stimulation and may underlie adaptation of systems in which TRPM4 is a key component.

Our description of the regulation of TRPM4 by PI(4,5)P₂ extends findings we reported for TRPM5, a functionally and structurally related ion channel (6). In these studies, we showed that TRPM5 currents run down in a Ca²⁺-dependent manner in excised inside-out patches and that rundown of TRPM5 is accompanied by a change in Ca²⁺ sensitivity (6) similar to what we now report for TRPM4. We also reported that TRPM5 currents could be recovered from desensitization by 10 μM diC₈-PI(4,5)P₂ (5), although in these experiments we never observed complete recovery of the currents. In our present studies, we frequently observed complete recovery of TRPM4 currents by 10 μM diC₈-PI(4,5)P₂. The difference in efficacy of PI(4,5)P₂ in recovering TRPM4
PI(4,5)P₂ Regulation of TRPM4

and TRPM5 currents suggests that there may be interesting differences in the functional control of these related channels. Most likely, either TRPM5 channels are less sensitive to PI(4,5)P₂ than are TRPM4 channels or they undergo a PI(4,5)P₂-insensitive form of rundown. However, caution should be exercised when comparing the efficacy of PI(4,5)P₂ for modulating different ion channels as efficacy may vary according to the conditions under which the channels are activated. For example, widely different EC₅₀ values for regulation of TRPM8 by PI(4,5)P₂ (ranging from ~5 to 100 μM) were reported depending on the relative activation of the channels by temperature, menthol, and voltage (21), a result that can be explained by an allosteric model for regulation of the channels. Similarly we expect that at different voltages or Ca²⁺ concentrations we will observe varying amounts of regulation of TRPM4 and TRPM5 by PI(4,5)P₂.

Our observation that TRPM4 was strongly regulated by PI(4,5)P₂ provides a mechanism for understanding the rundown of TRPM4 currents and recovery by MgATP first noted by Nilius et al. (22, 41) and Ulrich et al. (42). First we found that the Ca²⁺ sensitivity and voltage dependence of TRPM4 currents, both of which changed during rundown, could be fully restored by PI(4,5)P₂. This indicates that PI(4,5)P₂, or a similar molecule, is initially bound to the channels before rundown. Second we found that the recovery of TRPM4 currents by MgATP required magnesium and was sensitive to wortmannin, suggesting that it is mediated by a lipid kinase (28, 29). MgATP has been shown to restore activity of a number of PI(4,5)P₂-sensitive ion channels following rundown in excised patches, presumably by restoring levels of phosphorylated phosphatidylinositol (11, 12, 27). The sensitivity of this process to wortmannin and not LY294002 argues that this kinase is PI4K and not PI3K (31, 32), although definitive identification of key signaling molecules will require more complete molecular analysis of our expression system. Finally our data suggest that the initial desensitization of the channels is likely due to the activity of a membrane-associated, Ca²⁺-activated PLC. Such activity has been documented in a number of cell types (43, 44) and has been proposed to underlie rundown of a variety of other PI(4,5)P₂-sensitive ion channels, including TRPM8, TRPM5, and Kir6.2 (6, 20, 21, 30). These data point to the importance of the lipid biosynthetic machinery in regulating the activity of TRPM4.

The Ca²⁺ sensitivity of TRPM4 has been the subject of controversy with widely different half-activating concentrations reported by different laboratories (45). In excised patch recording mode where Ca²⁺ levels can be well controlled, we found an EC₅₀ for activation by Ca²⁺ of 110 μM immediately upon patch excision and 520 μM following desensitization; the latter value is similar to values of EC₅₀ reported for activation of native TRPM4-like channels (e.g. Refs. 25 and 46). We could not detect any significant current in response to 12 μM Ca²⁺ using HEDTA as the Ca²⁺ buffer even when the patch was directly excised into this solution (data not shown). In similar experiments, others have reported values of EC₅₀ of 4 μM immediately following patch excision and 140–170 μM after desensitization (41, 42). However, it should be noted that the former value was obtained from pooled patches with considerable measurement error (41) and that Ca²⁺ was buffered to micromolar concentrations with EGTA, which is well out of its range (47). In whole cell recording mode, TRPM4 currents were initially reported by Launay et al. (3) to have an apparent kᵢ of ~400 nM but later studies by Nilius et al. (22) and Ulrich et al. (42) showed that in recording mode mouse TRPM4 is half-activated by 15–20 μM Ca²⁺. The higher sensitivity to Ca²⁺ in whole cell recording mode suggests that components of the channel may be lost during patch excision. Hydrolysis or loss of PI(4,5)P₂ from the patch cannot explain this discrepancy because even in the presence of saturating concentrations of PI(4,5)P₂ we did not observe activation of TRPM4 in excised patches at such low Ca²⁺ concentrations. One possibility that cannot be excluded is that, in whole cell mode, Ca²⁺ levels in microdomains near the channels may be higher than expected based on the concentration of Ca²⁺ in the recording pipette and on the measurement of global Ca²⁺ levels with imaging techniques.

Our data suggest a novel mechanism by which PI(4,5)P₂ affects gating of TRP channels. It was shown recently that PI(4,5)P₂ shifts the voltage dependence of activation of TRPM8 (21), a channel that is structurally related to TRPM4 but is gated by cold and menthol (48, 49). We did not observe a significant shift in the midpoint for voltage-dependent activation of TRPM4 currents in response to PI(4,5)P₂ but instead observed a change in the fraction of the TRPM4 current that was voltage-sensitive. This is evident in the steady-state activation curves, which do not approach zero at very negative potentials. This voltage-insensitive component of the current was greatly increased by PI(4,5)P₂ exposure. Our data are accommodated by a model that we previously proposed to describe the gating of Ca²⁺-activated nonselective channels in more- 

onal sensory neurons (25). In this model, channels can open following Ca²⁺ binding to a low open probability (Pₒ) state, and voltage promotes transition to a high Pₛ state. Importantly and in contrast to a model previously proposed for activation of TRPM4 (41), our model predicts that currents can be activated at strongly negative membrane potentials. One possibility consistent with our data is that PI(4,5)P₂ acts to increase the open probability of the low Pₒ state, thus unblocking channel opening from voltage (see supplemental Fig. 2). The fact that we did not see a shift in the steady-state activation curve argues against a primary effect of PI(4,5)P₂ in stabilizing the high Pₛ state. Structurally one can imagine that the voltage sensor of the TRPM4 channel at normal resting potentials acts as a partial channel blocker and that block is relieved by movement of the voltage sensor in response to high membrane potentials or interaction of the voltage sensor with PI(4,5)P₂. This mechanism is consistent with a likely structural similarity between TRPM4 and voltage-gated K⁺ channels and the observation that the voltage sensor of the K⁺ channel lies at the lipid-protein interface where it is accessible to lipid modulation (50).

Is PI(4,5)P₂, an endogenous regulator of TRPM4? Although this question cannot be answered with certainty, several lines of evidence suggest that this is the case. First, although TRPM4 was equally sensitive to all doubly phosphorylated PIs, PI(4,5)P₂ is 100-fold more abundant than all doubly phosphorylated PIs combined (39). The triply phosphorylated PI, PI(3,4,5)P₃, was twice as effective as PI(4,5)P₂ but under non-stimulated conditions is present at ~100 the concentration of PI(4,5)P₂ (39). The only PI that is at concentrations comparable to PI(4,5)P₂ is PI(4)P, which was ineffective at regulating TRPM4. Moreover, based on our measured EC₅₀ of 5.8 μM for diCa²⁺-PI(4,5)P₂, we expect that at the normal resting membrane concentration of PI(4,5)P₂ (300 μM) (51) all channels will be fully bound. This is consistent with our observation that prior to desensitization TRPM4 channels were not sensitive to exogenous PI(4,5)P₂ and that following desensitization a saturating concentration of PI(4,5)P₂ fully restored the current.

Ca²⁺-activated cation channels were first described more than 30 years ago (52), and since then TRPM4-like channel activity has been noted in numerous cell types (10, 25, 53). Desensitization of native TRPM4-like channels has not been well described; this may be due to the fact that in many of these experiments the channels may have been inadvertently exposed to high Ca²⁺ solutions prior to testing (e.g. Ref. 25). In T lymphocytes, TRPM4 is thought to be gated by the Ca²⁺ elevation that occurs as a result of T cell receptor stimulation of the PLC.
signaling pathway (9). Our results suggest that activation of the T cell receptor and the consequent reduction of membrane PI(4,5)P₂ could, as a secondary effect, promote desensitization of TRPM4. Activation of TRPM4 leads to membrane depolarization (3), which limits the elevation of Ca²⁺ as the electrical driving force for Ca²⁺ entry is reduced (3, 9). The desensitization of TRPM4 is expected to have the opposite effect and thus to promote Ca²⁺ entry and elevation of intracellular Ca²⁺.

Thus fluctuations in PI(4,5)P₂ levels, through their effects on TRPM4, are likely to play a role in the complex signaling network that regulates the kinetics of Ca²⁺ oscillations in T cells. Whether PI(4,5)P₂ regulates this regulation is now be addressed.

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REFERENCES

1. Clapham, D. E. (2003) Nature 426, 517–524
2. Montell, C., Birnbaumer, L., and Flockerzi, V. (2002) Cell 108, 595–598
3. Launay, P., Fleig, A., Prenen, J., Droogmans, G., and Vassalli, D. (2002) Science 296, 1406–1407
4. Zhang, H., Craciun, L. C., Mirshahi, T., Rohacs, T., Lopes, C. M., Jin, T., and Logothetis, D. E. (2003) Neuron 37, 963–975
5. Huang, E. S., and Hille, B. (2002) Cell 109, 45–57
6. Wang, C., and Balla, T. (2001) J. Biol. Chem. 276, 14067–14070
7. Xie, L., Zhang, Z., and Balla, T. (2005) J. Biol. Chem. 280, 15045–15048
8. Rohacs, T., Lopes, C. M., Jin, T., and Logothetis, D. E. (2003) Nat. Neurosci. 6, 698–705
9. Liu, B., and Qin, F. (2005) J. Neurosci. 25, 1674–1681
10. Rohacs, T., Lopes, C. M., Michalidis, I., and Logothetis, D. E. (2005) Nat. Neurosci. 8, 626–634
11. Nilius, B., Prehn, J., and Nygren, R. (2002) J. Physiol. 548, 777–787
12. Nilius, B., Prehn, J., and Nygren, R. (2004) Pfluegers Arch. 448, 70–75
13. Hilgemann, D. W., and Ball, R. (1996) Science 273, 956–959
14. Zhang, H., Craciun, L. C., Mirshahi, T., Rohacs, T., Lopes, C. M., Jin, T., and Logothetis, D. E. (2003) Nature 426, 517–524
15. Runnels, L. W., Yue, L., and Clapham, D. E. (2002) Nat. Cell Biol. 4, 329–336
16. Hardie, R. C., Raghu, P., Moore, S., Juusola, M., Baines, R. A., and Sweeney, S. T. (2001) Neuron 30, 149–159
17. Liu, B., Zhang, C., and Qin, F. (2005) J. Neurosci. 25, 4835–4843
18. Liu, B., and Qin, F. (2005) J. Neurosci. 25, 1674–1681
19. Rohacs, T., Lopes, C. M., Michalidis, I., and Logothetis, D. E. (2005) Nat. Neurosci. 8, 626–634
20. Nilius, B., Prehn, J., and Nygren, R. (2002) J. Physiol. 548, 777–787
21. Liu, D., Zeng, Z., and Liman, E. R. (2005) J. Biol. Chem. 280, 20691–20699
22. Nilius, B., Prehn, J., and Nygren, R. (2004) Pfluegers Arch. 448, 70–75
23. Hardie, R. C., and Ball, R. (1996) Science 273, 956–959
24. Hilgemann, D. W., and Ball, R. (1996) Biochim. Biophys. Acta 514, 655–665
25. Nakanishi, S., and Kajihara, S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5317–5321
26. Vlahos, C., and Hille, B. (2002) Proc. Natl. Acad. Sci. U. S. A. 100, 5241–5248
27. Luo, V. D., Kaznachejeva, E., and Hille, B. (1998) Pfluegers Arch. 435, 595–602
28. Peier, A. M., Moqrich, A., Hergarden, A. C., Reeve, A. J., Andersson, D. A., and Story, M. V. (2002) Cell 110, 595–602
29. Balla, T. (2001) J. Cell Biol. 152, 956–959
30. Nakanishi, S., and Kajihara, S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5317–5321
31. Vlahos, C., and Hille, B. (2002) Proc. Natl. Acad. Sci. U. S. A. 100, 5241–5248
32. Luo, V. D., Kaznachejeva, E., and Hille, B. (1998) Pfluegers Arch. 435, 595–602
33. Partridge, L. D., and Swandulla, D. (1988) Trends. Neurosci. 11, 69–72
34. Ullrich, N. D., Voets, T., Prehn, J., and Nygren, R. (2002) J. Physiol. 548, 777–787
35. Runnels, L. W., Yue, L., and Clapham, D. E. (2002) Nat. Cell Biol. 4, 329–336
36. Hardie, R. C., Raghu, P., Moore, S., Juusola, M., Baines, R. A., and Sweeney, S. T. (2001) Neuron 30, 149–159
37. Liu, B., Zhang, C., and Qin, F. (2005) J. Neurosci. 25, 4835–4843
38. Liu, B., and Qin, F. (2005) J. Neurosci. 25, 1674–1681
39. Rohacs, T., Lopes, C. M., Michalidis, I., and Logothetis, D. E. (2005) Nat. Neurosci. 8, 626–634
40. Nilius, B., Prehn, J., and Nygren, R. (2002) J. Physiol. 548, 777–787
41. Liu, D., Zeng, Z., and Liman, E. R. (2005) J. Biol. Chem. 280, 20691–20699
42. Nilius, B., Prehn, J., and Nygren, R. (2004) Pfluegers Arch. 448, 70–75
43. Hilgemann, D. W., and Ball, R. (1996) Science 273, 956–959
44. Balla, T. (1996) Biochim. Biophys. Acta 514, 655–665
45. Nakanishi, S., and Kajihara, S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5317–5321
46. Vlahos, C., and Hille, B. (2002) Proc. Natl. Acad. Sci. U. S. A. 100, 5241–5248
47. Luo, V. D., Kaznachejeva, E., and Hille, B. (1998) Pfluegers Arch. 435, 595–602
48. Peier, A. M., Moqrich, A., Hergarden, A. C., Reeve, A. J., Andersson, D. A., and Story, M. V. (2002) Cell 110, 595–602
49. Balla, T. (2001) J. Cell Biol. 152, 956–959
50. Nakanishi, S., and Kajihara, S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5317–5321
51. Vlahos, C., and Hille, B. (2002) Proc. Natl. Acad. Sci. U. S. A. 100, 5241–5248
52. Luo, V. D., Kaznachejeva, E., and Hille, B. (1998) Pfluegers Arch. 435, 595–602
53. Partridge, L. D., and Swandulla, D. (1988) Trends. Neurosci. 11, 69–72