The Ca^{2+} -activated cation channel TRPM4 is regulated by phosphatidylinositol 4,5-bisphosphate

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Transient receptor potential (TRP) channel, melastatin subfamily (TRPM)4 is a Ca^{2+} -activated monovalent cation channel that depolarizes the plasma membrane and thereby modulates Ca^{2+} influx through Ca^{2+} -permeable pathways. A typical feature of TRPM4 is its rapid desensitization to intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$). Here we show that phosphatidylinositol 4,5-bisphosphate (PIP_2) counteracts desensitization to $[\text{Ca}^{2+}]_i$ in inside-out patches and run-down of TRPM4 currents in whole-cell patch-clamp experiments. PIP_2 shifted the voltage dependence of TRPM4 activation towards negative potentials and increased the channel's Ca^{2+} sensitivity 100-fold. Conversely, activation of the phospholipase C (PLC)-coupled M1 muscarinic receptor or pharmacological depletion of cellular PIP_2 potently inhibited currents through TRPM4. Neutralization of basic residues in a C-terminal pleckstrin homology (PH) domain accelerated TRPM4 current desensitization and strongly attenuated the effect of PIP_2 , whereas mutations to the C-terminal TRP box and TRP domain had no effect on the PIP_2 sensitivity. Our data demonstrate that PIP_2 is a strong positive modulator of TRPM4, and implicate the C-terminal PH domain in PIP_2 action. PLC-mediated PIP_2 breakdown may constitute a physiologically important brake on TRPM4 activity.

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Introduction

Phosphoinositides (PI) are ubiquitously used signaling molecules in eukaryotic cells. Phosphatidylinositol 4,5-bisphosphate (PIP_2) comprises about 3% of the total acidic membrane lipids and more than 99% of the doubly phosphorylated PI in a mammalian cell (McLaughlin *et al*, 2002). The list of ion channels that are regulated by PIP_2 includes inward-rectifier and voltage-gated K^+ channels, the two-P domain K^+ channels, voltage-gated Ca^{2+} channels, cyclic

nucleotide-gated channels, intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) release channels and the epithelial Na^+ and Cl^- channels (for a recent review, see Suh and Hille, 2005).

More recently, it was shown that several members of the transient receptor potential (TRP) channel superfamily are also regulated by PIP_2 . TRPV1 is tonically inhibited by PIP_2 (Chuang *et al*, 2001; Prescott and Julius, 2003), whereas TRP channel, melastatin subfamily (TRPM)5 (Liu and Liman, 2003), TRPM7 (Runnels *et al*, 2002), TRPM8 (Liu and Qin, 2005; Rohacs *et al*, 2005) and TRPV5 (Lee *et al*, 2005; Rohacs *et al*, 2005) are all activated in the presence of PIP_2 . Rohacs *et al* (2005) proposed a general role for the proximal C-terminal TRP domain in PIP_2 regulation of TRPM8 and other PIP_2 -activated TRP channels, whereas Prescott and Julius (2003) identified a more distal C-terminal region as a crucial determinant of PIP_2 inhibition.

TRPM4 is a widely expressed member of the melastatin subfamily of TRP channels. It functions as a Ca^{2+} -activated and voltage-dependent monovalent cation channel that depolarizes the plasma membrane and thereby modulates Ca^{2+} influx through Ca^{2+} -permeable pathways (Launay *et al*, 2002; Hofmann *et al*, 2003; Nilius *et al*, 2003). This physiological role was nicely illustrated in T-lymphocytes, where TRPM4 provides a negative feedback on Ca^{2+} entry, thereby allowing the Ca^{2+} oscillations that control T-cell cytokine release (Launay *et al*, 2004). A hallmark feature of TRPM4 is its rapid desensitization to $[\text{Ca}^{2+}]_i$ (Nilius *et al*, 2004; Zhang *et al*, 2005). The mechanisms that underlie this desensitization are poorly understood. It has been shown that the Ca^{2+} sensitivity of TRPM4 is regulated by ATP, PKC-dependent phosphorylation and by binding of calmodulin at the C-terminus (Nilius *et al*, 2005a).

Here we show that PIP_2 strongly enhances TRPM4 activity, by increasing the channel's Ca^{2+} sensitivity and shifting its voltage dependence of activation towards negative potentials. During revision of this article, the group of Liman (Zhang *et al*, 2005) published comparable observations. In addition, we provide complex mechanistic insights into the PIP_2 -dependent activation of the channel and present data that suggest a close interaction between TRPM4 and enzymes involved in the PIP_2 metabolism. Finally, we demonstrate that the TRP domain is not crucial for the PIP_2 effect on TRPM4, and identify positively charged amino acids in a C-terminal pleckstrin homology (PH) domain as important determinants of PIP_2 action. Thus, our data provide further evidence that PIP_2 is a general regulator of TRP channels, and implicate the C-terminal PH domain of TRPM4 in PIP_2 sensing.

Results

Effect of the phospholipase C inhibitor U73122 and PIP_2 on TRPM4 desensitization

A typical feature of whole-cell TRPM4 currents activated by high $[\text{Ca}^{2+}]_i$ is the rapid decay, which reflects a progressive decrease in the Ca^{2+} sensitivity of the channel (Figure 1A;

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Figure 1 Effects of the PLC blocker U73122 and PIP₂ on whole-cell currents through TRPM4. (A) Time course of the whole-cell currents after activation by 3 μM Ca²⁺ via the patch pipette. Shown are the currents obtained from voltage ramps at -100 (triangles) and +100 mV (circles), respectively. The IV curves at the right side panel are from the currents indicated by solid circles. Arrows mark the onset of the whole-cell mode. (B) Same experiment as in (A), but now in the presence of 10 μM U73122. Note the complete absence of desensitization and the nearly linear IV curves (right-side panel, current points are marked at the time course). (C) After desensitization, U73122 was applied to the bath. Note the slow recovery of the current traces (IV curves from ramps as in (A)). (D) Whole-cell configuration was established with 3 μM Ca²⁺ and 10 μM diC8-PIP₂ in the pipette. After initial desensitization, the currents completely recovered (traces as the right-hand side from the indicated points).

see also Hofmann *et al*, 2003; Nilius *et al*, 2003, 2004, 2005a). We first examined whether Ca²⁺-dependent activation of a phospholipase C (PLC) leading to PIP₂ depletion may underlie desensitization of TRPM4 (as suggested in Rohacs *et al*, 2005). Addition of the aminosteroid derivative U73122, an inhibitor of receptor-mediated PLC (Bleasdale *et al*, 1990; Smith *et al*, 1990), strongly attenuated the desensitization after activation by Ca²⁺ (Figure 1B). When U73122 was present in the extracellular solution before Ca²⁺-induced activation, the typical outward rectification of the current was also strongly reduced (Figure 1A and B, right panels). Application of U73122 after maximal current decay led to a significant reversal of the desensitization process (Figure 1C; $I_{\text{recovery}}/I_{\text{max}} = 0.72 \pm 0.13$, $n = 6$, measured at +100 mV). U73343, the homolog of U73122 that is ineffective on PLC, did not change desensitization of TRPM4 (data not shown). Furthermore, inclusion of the none-metabolizable PIP₂ diC8-PIP₂ (from hereon referred to as PIP₂) in the intracellular solution resulted in full recovery of the TRPM4 current after the initial decay (Figure 1D; $I_{\text{recovery}}/I_{\text{max}} = 1.06 \pm 0.14$, $n = 8$, +100 mV).

In inside-out patches, Ca²⁺-activated TRPM4 currents rapidly decay to a non-zero steady-state level, due to a time-dependent reduction of the channel's Ca²⁺ sensitivity (Nilius *et al*, 2004). With 100 μM Ca²⁺, currents at +100 mV decayed to approximately one-third of the maximal current after excision ($I_{\text{ss}}/I_{\text{max}} = 0.35 \pm 0.09$, $n = 5$; see Figure 2A).

Application of 10 μM PIP₂ to the cytosolic side of the inside-out patch resulted in a fast and full recovery of the current ($I_{\text{recovery}}/I_{\text{max}} = 1.07 \pm 0.15$, $n = 5$; Figure 2A) accompanied by an apparent loss of the voltage-dependent kinetics (Figure 2B). Recovery from desensitization by application of PIP₂ occurred with an EC₅₀ of 5.1 μM (Figure 2C and D), similar to the EC₅₀ reported elsewhere (Zhang *et al*, 2005). Washout of PIP₂ led to slow current decay to a level similar to that obtained for desensitization in the absence of PIP₂ ($I_{\text{ss}}/I_{\text{max}} = 0.28 \pm 0.11$, $n = 4$; compare Figure 2C and E). When 10 μM PIP₂ was applied to the bath before patch excision, desensitization was no longer observed ($I_{\text{ss}}/I_{\text{max}} = 0.97 \pm 0.09$, $n = 6$; Figure 2E). Likewise, blockade of PLC activity using 10 μM U73122 fully prevented desensitization of TRPM4 in inside-out patches ($I_{\text{ss}}/I_{\text{max}} = 0.95 \pm 0.07$, $n = 5$; Figure 2E), whereas U73343 was ineffective ($I_{\text{ss}}/I_{\text{max}} = 0.32 \pm 0.09$, $n = 3$). The effect of U73122 on the desensitization process was dose-dependent, with a concentration for half-maximal effect (EC₅₀) of 0.31 μM (Figure 2F).

When after maximal desensitization Ca²⁺ was removed and subsequently re-added, recovery was never observed. Likewise, U73122 was ineffective on desensitized inside-out patches (data not shown). However, when during the Ca²⁺-free period Mg-ATP was present at the cytosolic side, currents through TRPM4 recovered to nearly the same size as the maximal current after excision (Nilius *et al*, 2005a), and this

Figure 2 PIP₂ and U73122 counteract desensitization of TRPM4 in inside-out patches. (A) TRPM4 current measured at -100 and $+100$ mV using the step protocol in an inside-out patch excised in a solution containing $100 \mu\text{M}$ Ca^{2+} . Note that desensitization reaches a steady-state level. Application of $10 \mu\text{M}$ PIP₂ reverses the desensitization. (B) Current traces obtained at the time-points indicated in (A). Note the nearly complete disappearance of the time dependence after PIP₂ application. Same scaling as in figure (A). Protocol used to determine the dose dependence of the action of PIP₂ on TRPM4 (C) and resulting dose-response curve (D). The increase in outward current induced by a specific PIP₂ concentration was normalized to that obtained with $50 \mu\text{M}$ PIP₂. (E) PIP₂ and U73122 applied before patch excision prevent desensitization of TRPM4. (F) Dose-response curve for the effect of U73122 on TRPM4 desensitization. Each data point is from at least four independent measurements. Data points represent the ratio between the steady-state current and the maximal current measured immediately after application of Ca^{2+} .

recovery process was further potentiated by U73122 (Supplementary Figure S1). We conclude that Mg-ATP can reverse desensitization of TRPM4 by restoring the PIP₂ levels in the membrane patch. Indeed, Mg-ATP is known to activate phosphatidylinositol-4-kinases (PI-4-K), which regenerate PIP₂ from PI(4)P (Balla, 2001; Hilgemann *et al*, 2001). These results also indicate that both PI-4-K and PLC activities remain preserved in TRPM4-containing inside-out patches.

To determine whether other PI have a similar effect on TRPM4, we tested seven different PI-phosphates (Supplementary Figure S2). The following potency order was determined in inside-out patches ($300 \mu\text{M}$ $[\text{Ca}^{2+}]_i$): PI(4,5)P₂ > PI(3,4,5)P₃ = PI(3,5)P₂ = PI(3,4)P₂ = PI(5)P > PI(4)P = PI(3)P. Given that PIP₂ is both the most potent and the most abundant PI-phosphate, it is likely to represent the physiologically most important regulator of TRPM4.

It should be noted here that PIP₂ or U73122 did not have any effect on inside-out patches from nontransfected

cells or on TRPM4-containing patches in the absence of activating Ca^{2+} , excluding nonspecific actions of these compounds (data not shown). Moreover, we confirmed that $10 \mu\text{M}$ PIP₂ also caused recovery of TRPM4 endogenously expressed in umbilical vein-derived EA cells (Supplementary Figure S3).

PIP₂ and U73122 shift the voltage and Ca^{2+} dependence of TRPM4 activation

Many regulators of TRPM4 alter the voltage dependence of channel activation (Nilius *et al*, 2005a). Therefore, we examined the effects of PIP₂ and U73122 on the voltage dependence of TRPM4. In the absence of U73122 and PIP₂, hyperpolarizing voltage steps from a holding potential of 0 mV caused current deactivation, whereas activation was evident at positive potentials (Figure 3A). Nearly steady-state current-voltage (IV) relationships obtained at the end of 400 -ms voltage steps were fitted with a function that

Figure 3 PIP₂ modulates the voltage dependence of TRPM4 in inside-out patches. (A) Current measured in response to the indicated voltage protocol in the absence of PIP₂. (B) Voltage dependence of steady-state and tail current from the experiment shown in panel (A). (C) Voltage dependence of the open probability obtained from normalization of the tail current values to $I_{\max}$. (D–F) Same as panel (A–C), but now in the presence of 10 μ M PIP₂. (G) Activation curves for TRPM4 in control conditions and in the presence of either 10 μ M PIP₂ or 10 μ M U73122. Each data point is from 2–4 independent measurements. Solid lines represent fits using equation (3). (H) Average values for $V_{1/2}$ and z obtained from experiments as in panels (A–F).

combines a linear conductance multiplied by a Boltzmann activation term:

$$I = g * (V - E_{\text{rev}}) / (1 + \exp(-(V - V_{1/2})/s)) \quad (1)$$

where g is the whole-cell conductance, E_{rev} is the reversal potential, $V_{1/2}$ is the potential for half maximal activation and s is the slope factor. The slope factor s is related to the effective gating charge z of the voltage sensor according to

$$s = RT/zF \quad (2)$$

where R is the gas constant, T the absolute temperature and F the Faraday constant. As an alternative way to determine the voltage-dependent gating parameters, tail currents at -100 mV were measured and normalized to the maximal

tail current $I_{\max}$ (Figure 3A and B), yielding the voltage dependence of the steady-state open probability of TRPM4 (Figure 3C). Normalized tail currents were fitted using the equation

$$\frac{I}{I_{\max}} = \frac{1}{(1 + \exp(-(V - V_{1/2})/s))} \quad (3)$$

Equivalent values of s and $V_{1/2}$ were obtained by either method. As described in detail elsewhere, the s for TRPM4 is quite high, indicating that z is small (<1) (Figure 3; Nilius *et al*, 2005b).

Application of 10 μ M PIP₂ led to an almost complete loss of the time dependence of TRPM4 activation at positive

potentials, a dramatic slowing of current deactivation at negative voltages and significant steady-state inward currents (Figure 3D and E). The apparent steady-state open probability was shifted towards negative potentials and was shallower than in the absence of PIP₂ (Figure 3F–H). Virtually identical changes in voltage dependence were observed upon application of U73122 (Figure 3E and F and Supplementary Figure S4).

As described already in detail (Nilius *et al*, 2004), the decay of the TRPM4 current in inside-out patches is faster and more complete at low [Ca²⁺]_i, indicating that the Ca²⁺ sensitivity of the channel decreases after patch excision (Figure 4A). Inward currents are small (Figure 4B). Addition of 10 μM PIP₂ to the patch restored the Ca²⁺ sensitivity. Under persistent PIP₂ application, maximal TRPM4 current was already obtained at a [Ca²⁺]_i of 10 μM. Inward currents are large (note the relatively large currents at 0.5 μM [Ca²⁺]_i; Figure 4C and D). We quantified this effect by normalizing the steady-state current to the peak current obtained immediately upon patch excision in the different Ca²⁺ concentrations. The resulting values were fitted by a dose-response curve of the form:

$$I_{\text{norm}} = 1 / (1 + ([\text{Ca}^{2+}]_i / \text{EC}_{50})^{n_H}) \quad (4)$$

Figure 4 PIP₂ increases the Ca²⁺ sensitivity of TRPM4 in inside-out patches. (A) Representative time course of TRPM4 currents at –100 and +100 mV in an inside-out patch, illustrating the Ca²⁺ dependence of TRPM4 activation after desensitization. (B) Original traces from voltage steps obtained from different [Ca²⁺]_i indicated in (A). (C) Same as in (A), but in the presence of 10 μM PIP₂. (D) Original traces from voltage steps obtained from different [Ca²⁺]_i indicated in (C). (E) Dose dependence of TRPM4 activation by Ca²⁺ in the desensitized state and after addition of 10 μM PIP₂. The EC₅₀ value for Ca²⁺ activation changed from 134 μM (control, open circles, *n*_H = 0.9; see also Nilius *et al*, 2004) to 1.3 μM (*n*_H = 1.0).

where EC₅₀ represents the concentration for half-maximal TRPM4 activation and *n*_H the Hill coefficient. As shown in Figure 4E, application of 10 μM PIP₂ shifted the EC₅₀ value for Ca²⁺ from 134 ± 21 μM (control) to 1.3 ± 0.2 μM. A similar 10-fold increase in Ca²⁺ sensitivity was observed in the presence of U73122 (Figure 4E and Supplementary Figure S5). Interestingly, channel closure upon removal of Ca²⁺ was severely slowed down by PIP₂ and U73122. In the absence of these compounds, perfusion with an EGTA-containing Ca²⁺-free solution led to a virtually immediate inactivation of current (time constant < 1 s). In contrast, in the presence of PIP₂ or U73122, removal of Ca²⁺ led to a slow decay of the currents, with exponential time constants in the range of 6–20 s, *n* = 4 (see Supplementary Figure S6). This suggests that PIP₂ decreases the rate of Ca²⁺ unbinding from the channel.

Inhibition of TRPM4 by various PIP₂-depleting protocols

Wortmannin, an inhibitor of PI-4-K, retards the replenishment of PIP₂, leading to depletion of the intracellular PIP₂ pool (Nakanishi *et al*, 1995). In the whole-cell mode, inclusion of 50 μM wortmannin in the patch pipette reduced the amplitude of the Ca²⁺-activated current and led to a faster current decay (Figure 5A–C). Moreover, whole-cell TRPM4 currents were almost fully absent in cells pretreated for 25 min with 50 μM wortmannin (Figure 5D). Next, we tested whether the effect of wortmannin could be reversed by direct application of PIP₂. Indeed, after a 25-min pretreatment with 50 μM wortmannin, currents in inside-out patches ([Ca²⁺]_i = 300 μM) were strongly decreased (Figure 5E–G), and subsequent application of 10 μM PIP₂ led to a strong current increase (Figure 5E–G).

Poly-L-lysine (PLL) is a positively charged macromolecule that acts as a scavenger of PIP₂ (Lopes *et al*, 2002; Zhang *et al*, 2003). In inside-out patches, TRPM4 currents were rapidly inhibited when PLL was applied during the steady-state phase (Figure 5H and I). Inhibition by PLL was dose-dependent, and half-maximal inhibition was achieved at a concentration of 0.6 μg/ml (Figure 5J). TRPM4 currents were not restored upon washout of PLL, but subsequent application of PIP₂ led to partial reactivation of the channel (Figure 5H and K).

Inositol polyphosphate 5-phosphatases (5ptases) are enzymes that remove the phosphate group at the D5 position of PIP₂, leading to significant depletion of intracellular PIP₂ (Kisseleva *et al*, 2002). Using HEK cells with tetracyclin-inducible expression of 5ptase IV, we found that induction of this enzyme strongly reduces the peak and steady-state TRPM4 currents in inside-out patches (Supplementary Figure S7). Remarkably, application of PIP₂ reactivated TRPM4 currents in only ~40% of the induced cells, suggesting a high 5ptase activity in the close vicinity of TRPM4 channels.

Finally, we tested the effect of hormonal stimulation of PLC-coupled receptors on TRPM4 channel function. For this purpose, we used Chinese hamster ovary (CHO) cells permanently expressing the muscarinic receptor M1 (Buckley *et al*, 1989) and brief pulses of ionomycin (ION; 2 μM) to repetitively activate TRPM4 currents. In control conditions, a second pulse of ION applied 60–90 s after the first application led to a significant increase of the TRPM4 current at +100 mV (Figure 6A and C). In contrast, activation of the M1 receptor with 100 μM acetylcholine (ACh) after the first ION pulse led to a strong reduction of the current induced by

Figure 5 Wortmannin and poly-L-lysine attenuate currents through TRPM4 and reversion by PIP₂ in inside-out patches. (A) Time course of whole-cell currents measured as described in Figure 1 (circles + 100 mV, triangles – 100 mV). Activation of the current was established by 3 μ M $[\text{Ca}^{2+}]_i$ in the patch pipette. (B) Current activation as in (A). Wortmannin (WM, 50 μ M, same calibration as in (A)) was included in the patch solution. Note the much smaller currents and the faster desensitization as compared with (A). Preincubation with 50 μ M WM for 25 min completely prevented activation of TRPM4 currents with 3 μ M $[\text{Ca}^{2+}]_i$ (data not shown). (C) Pooled data from the maximal current obtained after breaking into the cells with 300 μ M $[\text{Ca}^{2+}]_i$ (supramaximal concentration for TRPM4; Ullrich *et al*, 2005). Note the decrease in the activated current by WM applied via the pipette and the nearly complete attenuation of the current for 25-min preincubation. (D) From the time courses of current activation with 300 μ M $[\text{Ca}^{2+}]_i$, the time to 50% desensitization was measured. This time was highly significantly shortened by application of WM into the pipette ($t_{1/2}$ values not measurable for 25-min preincubation). (E) Currents measured from inside-out patches (300 μ M $[\text{Ca}^{2+}]_i$). Before patch excision, cells were incubated for 25 min in 50 μ M WM. Note that the first current after excision is very small (see panel F). Application of PIP₂ restored the currents immediately. Typically, washing out of Ca^{2+} resulted in a delayed current decay (circles + 100 mV, triangles – 100 mV). Traces at the times indicated in the time course are shown at the right-hand side. (F) Averaged current size under control conditions (no WM preincubation, 300 μ M $[\text{Ca}^{2+}]_i$ measured at + 100 mV) and after 25-min preincubation with 50 μ M WM. (G) Comparison of the first current after excision (preincubation with WM) with the maximal current after 10 μ M PIP₂ application (cont: PIP₂ application without WM, currents measured at + 100 mV). (H) Time course of TRPM4 desensitization in inside-out patches (300 μ M $[\text{Ca}^{2+}]_i$). The time course at + 100 mV is shown (same step protocol as in (A)). Poly-L-lysine (PLL) is added to the inner side of the membrane in an inside-out patch. PLL (10 μ g/ml) already blocked the current completely. Reapplication of 10 μ M PIP₂ recovered the current to nearly the same size as the steady-state value. (I) Current traces for the indicated points in the time course shown in (H). (J) Concentration-response curve for the PLL block. Data were fitted by equation (4). The IC_{50} value is 0.6 μ g/ml ($n_H = 1.1$). (K) Recovery from desensitization measured from experiments as shown in (H), in which 10 μ M PIP₂ was applied after complete block by PLL. Steady-state current (e.g. trace b in panel H) was obtained from the stationary current divided by the maximal current ($I_{\text{ss}}/I_{\text{max}}$). The PIP₂ recovered current was measured by normalizing the maximal recovered current with the maximal current immediately after Ca^{2+} application, $I_{\text{recovery}}/I_{\text{max}}$. Both values are not statistically significant.

Figure 6 Activation of the M1 receptor inhibits TRPM4 in M1 receptor-expressing CHO cells. (A) Repetitive activation of TRPM4 by 2 μ M ION. The IV curves (right) were obtained at the indicated time points (left). (B) Same protocol as in (A), but 100 μ M ACh was applied between the first two ION applications. As a consequence, the second application of ION resulted in a much smaller current. A third ION application revealed partial recovery. (C) Averaged data from experiments as in (A) and (B). Current at +100 mV during the second ION application was normalized to that of the first ION application. The normalized current after a 3-min recovery period (recov) is also indicated. (D) Control currents in inside-out patches excised in 300 μ M $[Ca^{2+}]_i$. Traces indicated in the time course are shown at the right (the same current calibration). (E) Same as in (D), but now obtained from cells preincubated for 60 s with 100 μ M ACh. (F) Summary of the experiments on inside-out patches shown in (D) and (E). Both the peak currents (obtained immediately after patch excision) and steady-state currents were significantly decreased after preincubation with ACh ($P < 0.05$, $n = 12$). Shown are outward currents at +100 mV (columns up) and -100 mV (columns down).

the second ION pulse (Figure 6B and C). Under this condition, a third ION pulse applied 150–180 s after the washout of ACh revealed a partial recovery of the current (Figure 6B and C).

We also tested whether M1 activation before patch excision would affect the current size in inside out patches. A 60-s preincubation with 100 μ M ACh significantly reduced the first current measured after patch excision in 300 μ M $[Ca^{2+}]_i$ (Figure 6D–F). Additionally, desensitization of the currents in pretreated cells was faster and more complete than in untreated cells (compare Figure 6D and E). Thus, PLC-coupled receptor activation mimics the effects of PIP₂ depletion on TRPM4.

The C-terminal PH domain is a putative PIP₂ interacting site

Rohacs *et al* (2005) recently reported that mutating positively charged residues in the TRP box and TRP domain of TRPM8 led to a 10–100-fold reduction in the PIP₂ sensitivity. Moreover, equivalent mutations in the TRP domain of TRPM5 and TRPV5 increased the sensitivity of these channels to wortmannin, suggestive of a reduced PIP₂ sensitivity. They concluded that the TRP domain may be a general PIP₂-interacting site in different TRP channels. We investigated whether the corresponding mutations in TRPM4, K1059Q and

R1062Q in the TRP box and R1072Q in the TRP domain affect the response to PIP₂ in inside-out patches (Figure 7A). In comparison to WT TRPM4, R1062Q and R1072Q displayed a more complete desensitization, with steady-state outward current amplitudes that amounted to less than 5% of the peak current amplitude (Figure 8C–E), whereas K1059Q currents decayed to a similar steady-state level as WT TRPM4 (Figure 8A, B and E). Surprisingly, application of 10 μ M PIP₂ after maximal desensitization resulted in robust reactivation of all three mutants (Figure 8A–D). The degree of recovery, quantified as $I_{\text{recovery}}/I_{\text{max}}$, was not significantly different between WT TRPM4 and the three TRP domain mutations (Figure 8E). These data indicate that the TRP box and TRP domain are not the main determinants of PIP₂ action, and urged us to search for alternative PIP₂ interacting sites in TRPM4.

The C-terminus of TRPM4 contains two regions of positively charged residues that obey the consensus sequence of PH domains ([R/K]-X_{3–11}-[R/K]-X-[R/K]-[R/K], where X is any amino acid; see <http://us.expasy.org/prosite/>; Figure 7B), which are known as PIP₂ interaction sites (Harlan *et al*, 1994). The first, most proximal putative PH domain, R¹¹³⁶ARDKR¹¹⁴¹, was previously shown to determine modulation of TRPM4 by decavanadate (Nilius *et al*, 2004). Neutralization of all four basic residues in this region resulted

Figure 7 Schematic representation of mutations within putative PIP₂-binding regions of TRPM4. **(A)** Pairwise alignment of TRP domains of TRPM4 and TRPM5. Identical residues are shown in red within the yellow boxes, homologous residues in black within the green boxes. Note that the positively charged residues are conserved. Residues mutated in this study are marked by asterisks and are identical with the TRP box and TRP domain mutations used by Rohacs *et al* (2005). **(B)** Comparison of two putative PH domains in TRPM4 (dotted lines) with the corresponding region in TRPM5. Note the loss of the PH domains in TRPM5. Asterisks with numbers denote TRPM4 residues mutated in this work (for details, see Materials and methods).

in a mutant channel (termed ΔR/K) that exhibited fast and complete desensitization in inside-out patches (Figure 9A). Importantly, direct application of 10 μM PIP₂ never led to any current recovery ($n = 6$), whereas 30 μM PIP₂ induced to a small but significant recovery ($I_{\text{recovery}}/I_{\text{max}} = 0.29 \pm 0.1$; $n = 4$). Likewise, U73122 was unable to recover of the ΔR/K mutant. In contrast, charge-neutralizing mutations in the second putative PH domain did not affect the PIP₂ sensitivity of the channel, although these mutant channels exhibited significantly faster desensitization than WT TRPM4 (Figure 9B, C and E). From this, we conclude that the first putative PH domain is specifically involved in interaction with PIP₂ (Figure 9D and E).

As shown in Figure 7B, this first putative PH domain is not conserved in the closely related TRPM5. To test the consequence of this sequence divergence, we compared the PIP₂ sensitivity of TRPM4 with that of TRPM5, and of a chimera combining the N-terminus and transmembrane domains of TRPM4 with the C-terminus of TRPM5 (TRPM4/cM5 chimera). Excision in 300 μM [Ca²⁺]_i of inside-out patches from cells expressing TRPM4/cM5 led to robust current activation followed by nearly complete desensitization, which, however, is slower than for TRPM4 (Figure 9D and E; see also Liu and Liman, 2003; Ullrich *et al*, 2005). Application of 10 μM PIP₂ resulted in only a very small or no recovery of the current through TRPM4/cM5 chimera, compared to the complete recovery observed for TRPM4 under the same conditions (Figure 9D and E). Moreover, application of U73122 did not lead to any current recovery for TRPM5 or TRPM4/cM5 channels. Thus, the absence of the first PH domain in TRPM5 or the TRPM4/cM5 chimera correlates with the reduced PIP₂ sensitivity of these channels.

Discussion

Numerous intracellular signaling pathways depend on the activity of PLC, which hydrolyzes PIP₂ into membrane-bound

diacyl glycerol and soluble inositol trisphosphate, both potent intracellular signaling molecules. Moreover, PIP₂ itself acts as a regulator of signaling proteins in the plasma membrane, including a growing number of TRP channels (Chuang *et al*, 2001; Hardie *et al*, 2001; Runnels *et al*, 2002; Liu and Liman, 2003; Lee *et al*, 2005; Liu and Qin, 2005; Rohacs *et al*, 2005; Zhang *et al*, 2005). Although PIP₂ is the most abundant PI-phosphates, it has to be taken into account that all other PI-phosphates can also contribute to the modulation of TRPM4, as shown in Supplementary Figure S2. Compartmentalization and location with respect to the active channel will probably determine which PI-phosphate exerts the main effect. The action of PIP₂ on TRP channels can be either stimulatory, as is the case for TRPM5, TRPM7, TRPM8 and TRPV5 (Runnels *et al*, 2002; Liu and Liman, 2003; Lee *et al*, 2005; Rohacs *et al*, 2005), or inhibitory, as is the case for TRPV1 and *Drosophila* TRPL (Chuang *et al*, 2001; Estacion *et al*, 2001). Here we add the monovalent cation channel TRPM4 to the list of channels directly regulated by PIP₂. During revision of this article, qualitatively similar observations were published by Liman and colleagues (Zhang *et al*, 2005). In addition, we illustrate how TRPM4 activity can be modulated by several approaches that modify the cellular PIP₂ levels, including PLC-coupled M1 receptor activation, pharmacological inhibition of PLC activity, modulation of PIP₂ metabolism and scavenging of PIP₂. Finally, we present evidence that a distal C-terminal PH domain rather than the proximal C-terminal TRP domain is the major determinant of the PIP₂ sensitivity of TRPM4.

We found that PIP₂ is unable to gate TRPM4 when [Ca²⁺]_i is buffered at low levels, indicating that PIP₂ does not act as a direct TRPM4 activator. Instead, PIP₂ acts as a modulator of the channel's sensitivity to both Ca²⁺ and voltage: increasing PIP₂ levels cause a 100-fold increase in Ca²⁺ sensitivity and a drastic shift of the voltage dependence of activation towards negative potentials, thereby strongly increasing the open probability of TRPM4 at physiological membrane potentials. We propose that the decay of TRPM4 currents in whole-cell and inside-out patches, which entails a loss of sensitivity to both Ca²⁺ and voltage, reflects the gradual decrease of cellular PIP₂ levels due to a Ca²⁺-dependent stimulation of PLC activity and/or diffusional loss. A possible scheme to explain the combined effects of Ca²⁺, voltage and PIP₂ on TRPM4 activity is presented in Supplementary Figure S10.

The effects of PIP₂ on the voltage dependence of TRPM4 activation are reminiscent of its effects on the voltage sensitivity of voltage-gated channels from other channel families. For example, PIP₂ increases the open probability of TREK1 and KCNQ1 channels by shifting the activation curve to more negative voltages (Lopes *et al*, 2005). In P/Q-type Ca²⁺ channels, PIP₂ has a dual effect on channel activity (Wu *et al*, 2002). First, it shifts the activation curve of P/Q-type Ca²⁺ channels towards more positive potentials, thereby reducing channel opening at physiological potentials. Second, it causes a voltage-independent stabilization of these P/Q-type Ca²⁺ channels (Wu *et al*, 2002). In cloned and native N-type Ca²⁺ channels, PIP₂ strongly attenuated and reversed rundown of expressed channels (Gamper *et al*, 2004). Interestingly, we found that PIP₂ did not only shift the midpoint ($V_{1/2}$) of the activation curve of TRPM4 but also reduced its slope (s). This may suggest that PIP₂ reduces the effective gating charge, which could be explained by screening of voltage-sensing basic residues or by altering the

Figure 8 Effects of PIP₂ on TRP domain mutants in inside-out patches. (A) Time course showing WT TRPM4 current desensitization at -100 and $+100$ mV after excision in $300 \mu\text{M}$ $[\text{Ca}^{2+}]_i$ (same protocol as in Figure 2), followed by reversal of desensitization upon application of $10 \mu\text{M}$ PIP₂. To the right, raw current traces obtained at the indicated time points are shown using the same current calibration. (B–D) Same as in panel (A), but now for the indicated TRP domain mutants. (E) Averaged data showing the steady-state current before PIP₂ application (I_{ss}/I_{max}) and the current after PIP₂-induced recovery ($I_{recovery}/I_{max}$), both normalized to the maximal current immediately after excision. Note that even the completely desensitizing mutants show unchanged recovery with PIP₂.

electrical field around the voltage sensor (McLaughlin *et al*, 2002). Alternatively, PIP₂ may alter the coupling between the voltage sensor and the channel gating. Regardless of the mechanism, shifts of the voltage-dependent activation curve appear to represent a general activating principle whereby voltage-dependent TRP channels respond to thermal or chemical stimuli (Voets *et al*, 2004; Nilius *et al*, 2005b).

At present, we can only speculate about the mechanism whereby PIP₂ provokes a 100-fold increase in the apparent Ca²⁺ affinity of TRPM4. We found that, in the presence of PIP₂, washout of Ca²⁺ is dramatically slowed down, indicat-

ing that PIP₂ hinders the unbinding of Ca²⁺ from its activatory binding site. One possibility would be that the negatively charged phosphate groups of membrane-bound PIP₂ constitute an integral part of a high-affinity Ca²⁺-binding site. Loss of PIP₂ from the membrane would then result in a more rudimental, low-affinity Ca²⁺-binding site. Such a mechanism has, for example, been proposed to explain the increased Ca²⁺ affinity of synaptotagmin I, the putative Ca²⁺ sensor for rapid exocytosis, in the presence of PIP₂-containing phospholipid bilayers (Bai *et al*, 2004). Alternatively, PIP₂ binding may have an allosteric effect on TRPM4's Ca²⁺-binding site.

Figure 9 Effects of PIP₂ on putative PH domain mutants in inside-out patches. (A) Time course showing current desensitization at -100 and $+100$ mV for the $\Delta R/K$ mutant after excision in $300 \mu\text{M} [\text{Ca}^{2+}]_i$ (same protocol as in Figure 2), followed by partial reversal of desensitization upon application of $30 \mu\text{M}$ PIP₂. To the right, raw current traces obtained at the indicated time points are shown using the same current calibration. PIP₂ ($10 \mu\text{M}$), which was used in most other experiments, did not induce any recovery of this mutant ($n=6$, see E). (B) Same as (A), but now for the R1147Q mutant. Note the complete current recovery after application of $10 \mu\text{M}$ PIP₂. (C) Same as (A), but now for the RKR1147XAAA mutant. Note the small and rapidly desensitizing initial currents. In this case, application of $10 \mu\text{M}$ PIP₂ resulted in currents that were several-fold larger than the initial peak current (i.e. $I_{\text{recovery}}/I_{\text{max}} \gg 1$; see panel E). (D) Same as (A), but now for the TRPM4/cM5 chimeric channel. Note the slow desensitization and the negligible recovery upon application of PIP₂. (E) Averaged data showing the steady-state current before PIP₂ application ($I_{\text{ss}}/I_{\text{max}}$) and the current after PIP₂-induced recovery ($I_{\text{recovery}}/I_{\text{max}}$), both normalized to the maximal current immediately after excision. PIP₂ ($10 \mu\text{M}$) was used, unless noted otherwise.

In previous studies, two distinct PIP₂ interaction sites have been identified in TRP channels. First, a PIP₂ interaction site comprising eight positively charged residues was found in the C-terminus of TRPV1 (Prescott and Julius, 2003). Mutations

to these residues strongly reduced the tonic inhibition by PIP₂, leading to lowered thresholds for activation by heat or capsaicin. More recently, Rohacs *et al* (2005) identified three positive charges in the conserved TRP box and TRP domain

as determinants of the interaction between PIP₂ and TRPM8. Neutralization of these charges decreased the sensitivity of TRPM8 to activation by PIP₂ and aggravated the inhibitory effects of PIP₂ depletion. Equivalent mutations in TRPM5 and TRPV5 also increased the sensitivity of these channels to wortmannin, suggestive of a reduced PIP₂ sensitivity, although these mutant channels were not directly probed with PIP₂ (Rohacs *et al*, 2005). Surprisingly, when we made the corresponding mutations in the TRP domain of TRPM4, the activating effect of PIP₂ appeared fully conserved. This indicates that, at least in TRPM4, the TRP domain is not the main PIP₂ interaction site. We have to note, however, that two of the TRP domain mutants (R1062Q and R1072Q) exhibited accelerated desensitization, despite their normal PIP₂ sensitivity. Moreover, in these two cases, desensitization could not be prevented by U73122. One possible explanation for these results would be that these mutations do not interfere with PIP₂ binding, but rather alter the coupling between TRPM4 and PLC.

We identified two putative PH domains in the C-terminus of TRPM4, and implicated the first (i.e. closest to TM6) PH domain in PIP₂ activation. Neutralization of all four positively charged residues in this region resulted in a channel exhibiting very rapid desensitization and significantly reduced sensitivity to PIP₂. As this PH domain is not conserved in the closely related TRPM5, we constructed a chimeric channel consisting of the N-terminus and transmembrane domains of TRPM4 together with the C-terminus of TRPM5, and found that this TRPM4/cM5 channel also displayed desensitization similar to TRPM5 (Ullrich *et al*, 2005) and has an extremely reduced PIP₂ sensitivity. Interestingly, mutations to the first PH domain also abolish the effect of decavanadate on TRPM4 (Nilius *et al*, 2005a,b), indicating that PIP₂ and decavanadate act via a similar mechanism to alter the voltage- and Ca²⁺-sensitivity of TRPM4. In contrast, mutations to the second C-terminal PH domain were without effect on the channel's PIP₂ sensitivity.

Interestingly, several lines of evidence suggest that some of the enzymes involved in PIP₂ metabolism are tightly and functionally associated with TRPM4. Firstly, application of Ca²⁺-free, Mg-ATP-containing solution to desensitized TRPM4 in inside-out patches is able to fully reverse desensitization. This indicates that a PIP₂-regenerating kinase is present and functional in cell-free patches containing TRPM4. Secondly, the reversal of desensitization by Mg-ATP is potentiated by the PLC inhibitor U73122, suggesting that (Ca²⁺-dependent) PLC remains active in inside-out patches. Thirdly, some of the mutations to the TRP domain and to the second PH domain described in this work reduce the effect of PLC inhibition, without clear effects on PIP₂ sensitivity. This indicates that residues in TRPM4 may have an effect on PLC activity. Finally, overexpression of the PIP₂-depleting enzyme 5ptase IV not only led to lower TRPM4 activity in the inside-out patches but also reduced the effectiveness of exogenously applied PIP₂ to restore channel activity. This latter result could be explained assuming a concentrated 5ptase activity in the close vicinity of TRPM4 channels. It is interesting to speculate that TRPM4 in the plasma membrane forms part of a signaling complex with PLC, PI-4K and 5ptase, similar to the signalplex described in *Drosophila* rhabdomers, which consists of the cation channels TRP and TRPL together with components

of the signal transduction pathways, such as PLC, PKC and calmodulin.

In conclusion, we have demonstrated that PIP₂ enhances the activity of the Ca²⁺-activated cation channels TRPM4 by decreasing its Ca²⁺ sensitivity and shifting its voltage dependence, and provided evidence that these effects require an intact C-terminal PH domain. Activation of PLC-coupled receptors is expected to have a dual effect on TRPM4 activity. On the one hand, PLC activity will lead to the production of IP₃, which releases Ca²⁺ from intracellular stores, leading to a rise in [Ca²⁺]_i and activation of TRPM4. On the other hand, PLC-dependent hydrolysis of PIP₂ will cause desensitization of TRPM4, thereby limiting its activity. PIP₂-dependent signaling emerges as a general mechanism for the regulation of diverse TRP channel functions.

Materials and methods

Cell culture

Human embryonic kidney (HEK) 293 cells and CHO cells expressing the human muscarinic M1 receptor were cultured as described in detail previously (Buckley *et al*, 1989). We used a T-Rex tetracyclin-regulated system (Invitrogen) for inducible expression of 5ptase IV in HEK293 cells (Kisseleva *et al*, 2002; Voets *et al*, 2004; Verbsky *et al*, 2005). To induce expression of 5ptase IV, 0.1 µg/ml tetracycline was added to the culture medium, and cells were used 12–16 h after induction. For transient expression of TRPM4, we used the bicistronic expression plasmid pdiTRPM4b, which carries the entire coding region for the human TRPM4b (accession number AX443227, kindly provided by Drs V Flockerzi and U Wissenbach) and for the green fluorescent protein, coupled with an internal ribosomal entry site sequence. HEK293 and CHO cells were transfected using *TransIT*-293 transfection reagent (Mirus Corp., Madison, WI) and successfully transfected cells were identified by their green fluorescence (for details, see Nilius *et al*, 2003). Mutations were made using the standard polymerase chain reaction overlap extension technique (Ho *et al*, 1989).

Solutions

The extracellular solution for cell attached measurements and the pipette solution for inside-out patch clamp measurements contained (mM): 156 NaCl, 5 CaCl₂, 1 MgCl₂, 10 N-(hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), buffered at pH 7.4 with NaOH. Inside-out patches were excised in a solution containing (mM): 156 CsCl, 1 mM MgCl₂, 10 HEPES, pH 7.2 with CsOH. The Ca²⁺ concentration at the inner side of the membrane was adjusted between 100 nM and 5 µM by adding appropriate amounts of CaCl₂ calculated by the CaBuf program (<ftp://ftp.cc.kuleuven.be/pub/droogmans/cabuf.zip>) to the 5 mM ethylene-glycol-O,O'-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA)-containing solution. For Ca²⁺ concentrations between 10 µM and 1 mM, CaCl₂ was added to an EGTA-free solution. In all figures, '0Ca²⁺' refers to a Ca²⁺-free solution containing 5 mM EGTA. All internal solutions were ATP-free. Experiments were performed at room temperature (22–25°C). DiC8-PIP₂ was purchased from Echelon Biosciences (Salt Lake City, UT) and wortmannin, PLL, U73343 and U73122 from Sigma (St Louis, MO).

Electrophysiology

Currents were measured in the whole-cell and inside-out configuration using an EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany). Patch electrodes had a DC resistance between 2 and 4 MΩ when filled with the different recording solutions. Currents were sampled at 2.5 kHz and filtered at 1 kHz. The step protocols consisted of a 400-ms step to –100 mV from a holding of 0 mV, followed by a 250-ms step to +100 mV applied at 0.5 Hz. The ramp protocol consisted of a 400-ms ramp from –100 to +100 mV from a holding potential of 0 mV.

Data analysis

Electrophysiological data were analyzed using the WinASCD software (G Droogmans, Leuven, Belgium). Pooled data are given

as mean $\pm$ s.e.m. of n cells. Significance was tested using Student's paired t -test ($P < 0.05$).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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