Phosphatidylinositol 4,5-bisphosphate (PIP$_2$) and Ca$^{2+}$ are both required to open the Cl$^-$ channel TMEM16A

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Transmembrane member 16A (TMEM16A) is a widely expressed Ca$^{2+}$-activated Cl$^-$ channel with various physiological functions ranging from mucosal secretion to regulating smooth muscle contraction. Understanding how TMEM16A controls these physiological processes and how its dysregulation may cause disease requires a detailed understanding of how cellular processes and second messengers alter TMEM16A channel gating. Here we assessed the regulation of TMEM16A gating by recording Ca$^{2+}$-evoked Cl$^-$ currents conducted by endogenous TMEM16A channels expressed in Xenopus laevis oocytes, using the inside-out configuration of the patch clamp technique. During continuous application of Ca$^{2+}$, we found that TMEM16A-conducted currents decay shortly after patch excision. Such current rundown is common among channels regulated by phosphatidylinositol 4,5-bisphosphate (PIP$_2$). Thus, we sought to investigate a possible role of PIP$_2$ in TMEM16A gating. Consistently, synthetic PIP$_2$ rescued the current after rundown, and the application of PIP$_2$ modulating agents altered the speed kinetics of TMEM16A current rundown. First, two PIP$_2$ sequestering agents, neomycin and anti-PIP$_2$, applied to the intracellular surface of excised patches sped up TMEM16A current rundown to nearly twice as fast. Conversely, rephosphorylation of phosphatidylinositol (PI) derivatives into PIP$_2$ using Mg-ATP or inhibiting dephosphorylation of PIP$_2$ using β-glycerophosphate slowed rundown by nearly 3-fold. Our results reveal that TMEM16A regulation is more complicated than it initially appeared; not only is Ca$^{2+}$ necessary to signal TMEM16A opening, but PIP$_2$ is also required. These findings improve our understanding of how the dysregulation of these pathways may lead to disease and suggest that targeting these pathways could have utility for potential therapies.

Transmembrane protein 16A (TMEM16A) (also known as ANO1, DOG1, ORAOV2, and TAOS2) is a Ca$^{2+}$-activated Cl$^-$ channel common to evolutionarily diverse organisms. The channel plays functionally varied roles including signaling contraction in cardiovascular smooth muscle cells (2), facilitating transepithelial water transport vital for mucociliary clearance in pulmonary epithelial cells (3), and transmitting pain detection in sensory neurons (as reviewed in Ref. 4). Disrupting TMEM16A function has serious implications exemplified by the perinatal lethality phenotype in TMEM16A-null mice (5). As a key regulator in multiple physiological processes, this channel could be a target for novel therapeutics to treat chronic conditions such as hypertension or cystic fibrosis.

Despite being identified only 10 years ago (6–8), several structural and functional studies revealed a wealth of information regarding how TMEM16A operates. To date, TMEM16A is known to be activated by elevated intracellular Ca$^{2+}$ (6–8) which binds to a membrane-embedded domain located near a Cl$^-$-conducting pore (9, 10). The functional TMEM16A channel is a dimer comprised of two identical subunits that each have 10 transmembrane domains and an independent Cl$^-$-conducting pore (11, 12). When Ca$^{2+}$ binds its cognate domain on TMEM16A, it induces a conformational rearrangement of the α-helix in the sixth transmembrane domain that physically opens the anion-conducting pore (10). Each Ca$^{2+}$-binding site in the TMEM16A channel can accommodate two Ca$^{2+}$ cations (13), and it appears as though the channel gates differently depending on whether one or two cations are bound (14). Intriguingly, the recent cryo-EM structures of TMEM16A in a Ca$^{2+}$-bound state revealed that the anion pore was not wide enough for Cl$^-$ permeation (9, 10), suggesting Ca$^{2+}$ alone may not be sufficient to activate TMEM16A channels. Another signaling molecule that may regulate TMEM16A is the acidic phospholipid phosphatidyl 4,5-bisphosphate (PIP$_2$) (15–17).

PIP$_2$ is a minor component of the membrane, yet it is a master regulator of membrane function (18). Importantly, PIP$_2$ serves as the substrate for phospholipase C cleavage to produce inositol trisphosphate (IP$_3$) and diacylglycerol; pathways that transduce extracellular signals to intracellular signaling events. However, PIP$_2$ also serves as a signaling molecule in its own right by regulating endocytosis and exocytosis, actin polymerization, establishment of basolateral polarity, and regulation of ion channels (19). A possible role for PIP$_2$ in TMEM16A regulation has already been explored by other groups, however, the interpretation of how PIP$_2$ alters TMEM16A currents remains disputed. One study reported that PIP$_2$ inhibits TMEM16A (16), another states that it does not regulate the channel (20), and yet two others indicate that PIP$_2$ promotes TMEM16A...
activity (15, 17). We speculate that the disparity among the experimental interpretations may stem from the use of indirect methods.

The objective of the research reported in this manuscript was to determine whether the phospholipid PIP₂ regulates TMEM16A channels. For these experiments, we made electrophysiology recordings from Xenopus laevis oocytes, cells that endogenously and abundantly express TMEM16A channels (7). Using excised inside-out patch clamp together with methods that directly alter the available PIP₂ content of the patch, we found that TMEM16A currents decayed following patch excision. By changing the available PIP₂ content, we altered the kinetics of this rundown. Moreover, depleting the membrane PIP₂ rendered TMEM16A channels unable to conduct current even in saturating concentrations of intracellular Ca²⁺. Together these findings establish that TMEM16A channels are potentiated by PIP₂.

Results

**TMEM16A currents recorded from excised inside-out patches decayed over time**

To study TMEM16A currents, we made recordings of the endogenous channel in X. laevis oocytes using the inside-out configuration of the patch clamp technique. Notably, the prominent Ca²⁺-activated current recorded from these cells is conducted by TMEM16A channels (7). During 100–150–ms steps to −60 and +60 mV, we observed robust, Ca²⁺-activated TMEM16A currents at both voltages (Fig. 1A). Surprisingly, these currents decayed over time despite the continued presence of a saturating concentration of intracellular Ca²⁺ (Fig. 1B) (9). Fig. 1A depicts typical currents recorded at −60 and +60 mV, at 30, 60, 120, and 180 s following 2 mM Ca²⁺ application. Loss of current from excised patches is a phenomenon known as rundown (21). We next sought to characterize the rundown of TMEM16A-conducted currents after patch excision.

We first explored whether the rundown of TMEM16A currents was voltage dependent. To do so, plots of the currents recorded during steps to −60 mV or +60 mV, versus time, were fit with single exponential functions and enabled us to quantify the kinetics of rundown (Equation 1) (Fig. 1C). In 15 independent experimental trials, the average rate of rundown was 68.9 ± 7.1 s at −60 mV, and 63.8 ± 5.9 s at +60 mV (Table 1 and Fig. 1D). Similar kinetics measured at the two voltages reveal that the current decay is voltage independent.

To explore a possible relationship between the rate of rundown and the number of channels in an excised patch, we plotted the rate of current decay recorded at −60 mV versus the peak steady state current (Fig. S1). In 15 independent trials, we found that there was no apparent relationship between these two metrics. These data suggest that TMEM16A currents ran down regardless of pipette size or the number of channels opened by Ca²⁺ across the different trials.

**PIP₂ recovered TMEM16A currents in inside-out excised patches**

Rundown of currents recorded in the inside-out configuration of the patch clamp technique is characteristic of channels regulated by PIP₂ (19). We therefore hypothesized that if TMEM16A current rundown occurred because of the depletion of PIP₂ in excised patches, exogenous PIP₂ application should recover TMEM16A currents following their decay. To do so, we applied a water-soluble analog of PIP₂ (22–25), dicotanylglycerol-PIP₂ (diC₈-PIP₂) to inside-out patches excised from X. laevis oocytes (Fig. 2A). For these experiments, we recorded TMEM16A-conducted currents at −60 mV before and during the application of 2 mM Ca²⁺. Once the Ca²⁺-activated currents ran down to a steady state, we applied 100 µM diC₈-PIP₂ (17) in the presence of 2 mM Ca²⁺. Fig. 2B depicts an example plot of TMEM16A-conducted currents recorded at −60 mV versus time, before and during 100 µM diC₈-PIP₂.

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**Table 1**

| Condition | Concentration | Tau, t (s) |
|-----------|---------------|------------|
| Control (Ca²⁺) | 2 mM | 68.9 ± 7.1 (n = 15) |
| Anti-PIP₂ | 15 µg/ml | 48.6 ± 7.4 (n = 4) |
| Neomycin | 50 mM | 53.2 ± 8.3 (n = 5) |
| Mg-AMP | 1.5 mM | 143.4 ± 12.8 (n = 5) |
| βGIP | 50 mM | 166.8 ± 14.7 (n = 5) |

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**Figure 1.** TMEM16A Ca²⁺-evoked Cl⁻ currents rundown in excised inside-out patches. Inside-out patch clamp recordings were conducted on macropatches excised from X. laevis oocytes. A, example currents recorded during 100–150–ms steps to −60 and +60 mV, at indicated times following patch excision. B, representative plot of current measured at −60 (bottom) or +60 mV (top) versus time, after patch excision. 2 mM Ca²⁺ was applied at 10 s as denoted by the gray bar. The dashed gray line represents 0 nanoamperes (nA). C, normalized plot of current measured at −60 mV versus time, fit with a single exponential (red line). D, box plot distribution of the rate of current decay (t), measured by fitting plots of relative current versus time with single exponentials (n = 15). The central line denotes the median, the box denotes the distribution of 25–75% of the data, and the whiskers represent 10–90% of the data. The t at +60 mV and −60 mV were not significantly different (p = 0.29) as determined by t test.

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**Figure 2.** A. Representative plot of TMEM16A-conducted currents after patch excision. B. Representative plot of TMEM16A-conducted currents after patch excision. C. Box plot distribution of the rate of current decay (t), measured by fitting plots of relative current versus time with single exponentials (n = 15). The central line denotes the median, the box denotes the distribution of 25–75% of the data, and the whiskers represent 10–90% of the data. The t at +60 mV and −60 mV were not significantly different (p = 0.29) as determined by t test.

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**Figure 3.** A. Example plot of TMEM16A-conducted currents recorded at −60 mV versus time, before and during 100 µM diC₈-PIP₂.
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Figure 2. PIP$_2$ analog recovered TMEM16A-conducted currents following rundown when applied with intracellular Ca$^{2+}$. The soluble synthetic analog of PIP$_2$, diC8-PIP$_2$, was applied to excised inside-out patches once current had stably run down. Currents were recorded at $-60$ mV.

Application of diC8-PIP$_2$ in the absence of Ca$^{2+}$, changing the current by 1.1 fold, was sufficient to recover TMEM16A currents only in the presence of intracellular Ca$^{2+}$ (Fig. S2, A and B). Surprisingly, we observed that natural PIP$_2$ recovered an average 28% of the peak current, which was less than the current recovered by 100 μM diC8-PIP$_2$. We proceeded with further experimentation only using the diC8-PIP$_2$. Next, we conducted additional TMEM16A current recovery experiments using 10 μM diC8-PIP$_2$ (Fig. S2C) and 30 μM diC8-PIP$_2$ (Fig. S2D). We observed that 10 μM or 30 μM diC8-PIP$_2$ incompletely recovered the current compared with 100 μM diC8-PIP$_2$ (Fig. 2C). Altogether, the data suggest that PIP$_2$ recovery of TMEM16A currents and that the currents are recovered in a concentration-dependent manner.

After demonstrating that PIP$_2$ recovers TMEM16A currents, we sought to further characterize the relationship between PIP$_2$ and TMEM16A. We tested whether 100 μM diC8-PIP$_2$ (Fig. 3A) was sufficient to recover TMEM16A current following rundown, or if diC8-PIP$_2$ and Ca$^{2+}$ were both required. Application of diC8-PIP$_2$ in the absence of Ca$^{2+}$ had a nominal effect on TMEM16A, changing the current by 1.1 ± 0.1-fold ($n = 5$) (Fig. 3, B and C). These data demonstrate that although Ca$^{2+}$ activates TMEM16A, PIP$_2$ is required for these channels to conduct Cl$^{-}$ currents. Moreover, PIP$_2$ potentiates TMEM16A currents only in the presence of intracellular Ca$^{2+}$.

The diC8-PIP$_2$ compound could theoretically regulate TMEM16A channels by interactions mediated by its lipid tail group or its phosphoinositid head group. Thus, we tested the hypothesis that the diacetylglycerol-phosphoinositid backbone (diC8-PI) (Fig. 3A) alone was sufficient to recover current. Following TMEM16A current rundown, we applied 100 μM diC8-PI with Ca$^{2+}$ and quantified the proportion of current recovered. Fig. 3D shows an example plot of TMEM16A currents recorded at $-60$ mV versus time, before and during diC8-PI application. In six separate trials, we observed that 100 μM diC8-PI application nominally altered the TMEM16A currents, recovering 1.4 ± 0.2-fold current, which was significantly lower than the 3.6 ± 0.5-fold current recovered by diC8-PIP$_2$ with Ca$^{2+}$ but not significantly different from the current recovered by diC8-PI in the absence of Ca$^{2+}$ (Fig. 3B). This result demonstrates that without the phosphorylated head groups, diC8-PI was unable to recover TMEM16A currents, thereby suggesting that the phosphates on the inositol head group mediate the TMEM16A-PIP$_2$ interaction. To determine the role of the phosphate head group in mediating the TMEM16A-PIP$_2$ interaction, we also applied 100 μM diC8-PI(4)P (Fig. 3A) with Ca$^{2+}$ and quantified the proportion of

**Figure 3.** PIP$_2$ and Ca$^{2+}$ are both required for TMEM16A-conducted currents. diC8-PIP$_2$, PI(diC8-PI), and PI(4)P(diC8-PI(4)P) were applied to excised inside-out patches once current had stably run down. Currents were recorded at $-60$ mV. A, schematic depiction of the various diC8 analogs used. B, box plot distribution of the fold current recovered after the application of diC8-PIP$_2$ with Ca$^{2+}$, diC8-PI, without Ca$^{2+}$, or diC8-PI(4)P with Ca$^{2+}$. The fold change in current recovered was calculated as change in current upon application of diC8-PIP$_2$ with Ca$^{2+}$. The data were analyzed with a one-way ANOVA, followed by a Dunnett’s multiple comparison test. C–E, representative plots of normalized currents versus time, before and during application of 100 μM diC8-PIP$_2$, with no added Ca$^{2+}$ (C), 100 μM diC8-PI with 2 mM Ca$^{2+}$ (D), or 100 μM diC8-PI(4)P with 2 mM Ca$^{2+}$ (E). ** denotes $p < 0.01$ and * denotes $p < 0.05$ as determined by t test. -Fold current recovered by diC8-PI without Ca$^{2+}$ compared with diC8-PI was not significantly different ($p = 0.20$).
current recovered. Fig. 3E shows an example plot of TMEM16A currents recorded at −60 mV versus time, before and during diC8-PI(4)P application. In nine separate trials, we observed that 100 μM diC8-PI(4)P application recovered 2.1 ± 0.2-fold current (Fig. 3B), which represented an average of 13% of the peak current. The intermediate recovery (lying between 3.6 ± 0.5-fold current recovered by diC8-PI(4)P with Ca$^{2+}$ and 1.4 ± 0.2-fold current recovered by diC8-PI with Ca$^{2+}$) suggests that the phosphate head group is important for mediating TMEM16A-PIP$_2$ interaction. Altogether, the data reveal that in addition to Ca$^{2+}$, PIP$_2$ regulates TMEM16A gating.

### Scavenging PIP$_2$ sped up TMEM16A current rundown

Our finding that diC8-PIP$_2$ restored TMEM16A currents following rundown suggested that rundown may be the result of PIP$_2$ depletion in excised patches. Thus, we reasoned that we should be able to speed up rundown by applying compounds that compete with TMEM16A for binding to PIP$_2$. We tested this hypothesis by quantifying the rate of TMEM16A current decay during application of two compounds known to scavenge PIP$_2$: A PIP$_2$-targeting antibody (anti-PIP$_2$) (26) and neomycin (27, 28) (Fig. 4A). We began these experiments by recording TMEM16A currents at −60 mV before and during treatment with 2 mM Ca$^{2+}$ applied with 15 μg/ml anti-PIP$_2$. In four independent trials, we observed that TMEM16A current ran down with an average rate of 48.6 ± 7.4 s from the presence of anti-PIP$_2$ compared with 68.9 ± 7.3 s from patches treated with 2 mM Ca$^{2+}$ alone (n = 15) (Fig. 4, B and C and Table 1).

In a parallel series of experiments, we quantified the rate of TMEM16A current rundown in the presence of the PIP$_2$ scavenger neomycin. Neomycin is an antibiotic that also scavenges PIP$_2$ when applied to the intracellular membrane at high concentrations (27). Fig. 4D depicts an example plot of the TMEM16A currents recorded at −60 mV versus time, before and during 2 mM Ca$^{2+}$ and 50 mM neomycin application. In five independent trials, neomycin sped up current rundown from 68.9 ± 7.3 s to 32.0 ± 8.8 s (Fig. 4B).

Altogether, we observed that both anti-PIP$_2$ and neomycin application sped up TMEM16A rundown (Fig. 4). Moreover, the rate of rundown was not significantly different in the presence of either anti-PIP$_2$ or neomycin (Fig. 4B and Table 1) consistent with the hypothesis that each compound was acting by scavenging PIP$_2$ rather than exerting nonspecific effects on the channel. Together, these data support the hypothesis that PIP$_2$ is required for TMEM16A to conduct Cl$^-$ currents.

### Slowing PIP$_2$ depletion slowed TMEM16A rundown

Phosphatases and kinases work together to maintain stable levels of PIP$_2$ in whole cells (Fig. 5A) (29). In excised patches, however, membrane-anchored kinases lack access to the ATP required to fuel phosphorylation and regeneration of PIP$_2$ (19, 29). Consequently, continued activity of phosphatases without counteracting kinases leads to PIP$_2$ dephosphorylation. We reasoned that if TMEM16A currents decayed in excised patches because of the dephosphorylation of PIP$_2$, then enabling rephosphorylation or inhibiting phosphatases should slow current loss. We tested the hypothesis by determining whether enabling rephosphorylation of PIP$_2$, with application of magnesium-adenosine triphosphate (Mg-ATP) (30, 31) would slow TMEM16A current rundown in excised inside-out patches. As a control for these experiments, we first recorded TMEM16A currents at −60 mV before and during the application of magnesium adenosine monophosphate (Mg-AMP), which can bind to, but not activate, kinases. Thus, Mg-AMP application should not affect current rundown. Indeed, we observed no difference in current rundown between Mg-AMP and control condition of 2 mM Ca$^{2+}$ (Fig. 5, B and C). We then recorded TMEM16A currents at −60 mV before and during application of 2 mM Ca$^{2+}$ with 1.5 mM Mg-ATP. An example plot of normalized current versus time is shown in Fig. 5D. We observed that in the presence of 1.5 mM Mg-ATP and 2 mM Ca$^{2+}$, TMEM16A currents ran down over a longer time course, with a time constant of 143.4 ± 17.2 s (n = 5) (Fig. 5D and Table 1).

Next, we reasoned that if phosphatase-mediated PIP$_2$ depletion causes TMEM16A current rundown in excised patches then inhibiting phosphatase activity would also slow rundown. We tested this hypothesis by quantifying the kinetics of current rundown in the presence of Ca$^{2+}$ and the general phosphatase inhibitor sodium β-glycerophosphate pentahydrate (βGP) (32). Fig. 5E depicts an example plot of TMEM16A-conducted cur-
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![Diagram](image)

**Figure 5.** Enabling rephosphorylation or inhibiting phosphatases slowed current decay in excised inside-out patches. A, schematic depicting the role of phosphatases and kinases in generating various phosphoinositide species. B, the box plot distribution of the rate of rundown (t) observed from currents recorded at −60 mV from inside-out patches exposed to 2 mM Ca²⁺ with 1.5 mM Mg-AMP (n = 5), 2 mM Ca²⁺ with 1.5 mM Mg-ATP (n = 6), and 2 mM Ca²⁺ with 50 mM sodium βGP (n = 6). Single exponentials were fitted to current traces to obtain the rate of current rundown. Solid gray line denotes median rate of rundown measured from patches recorded under the control conditions of 2 mM Ca²⁺-only application, and the dashed lines represent the control data distribution from 25 to 75%. C–E, representative plots of normalized currents versus time made before and during application of 1.5 mM Mg-AMP with 2 mM Ca²⁺ (compared with 2 mM Mg²⁺-only application with the gray dashed line) (C), 1.5 mM Mg-ATP with 2 mM Ca²⁺ (compared with 1.5 mM Mg-AMP with the orange dashed line) (D), and 50 mM βGP with 2 mM Ca²⁺ (compared with 2 mM Ca²⁺-only application with the gray dashed line) (E). ** denotes p < 0.01 and * denotes p < 0.05 when compared with 2 mM Ca²⁺-only application or between indicated pairs determined by t test.

Currents recorded at −60 mV versus time, before and during application of 50 mM βGP and 2 mM Ca²⁺. In five independent trials, we observed that TMEM16A currents ran down slower in the presence of the phosphatase inhibitor, with a time constant of 166 ± 14.7 s (n = 5) (Fig. 5E and Table 1). Together, these data suggest that TMEM16A currents run down in excised patches as the result of PIP₂ depletion via its dephosphorylation. Moreover, these data are consistent with the hypothesis that the phosphates found on the inositol head group of PIP₂ are responsible for the interaction between TMEM16A and PIP₂.

**Discussion**

By recording TMEM16A currents while modifying the membrane PIP₂ content, here we demonstrate that these channels require both Ca²⁺ and PIP₂ to conduct current. A possible role for PIP₂ in TMEM16A regulation was initially suggested by rundown of these currents recorded from *X. laevis* oocytes despite the continued presence of a saturating concentration of Ca²⁺ (Fig. 1). Rundown results from the removal of a membrane patch from the cytosol that includes ATP (29). This ATP is required to fuel the phospholipid kinases which rephosphorylate the phospholipids present in the membrane (29). As such, current rundown in excised patches is a hallmark of channels regulated by PIP₂.

Application of the soluble PIP₂ analog diC₈-PIP₂ recovered TMEM16A-conducted currents following rundown, but only when applied with Ca²⁺ (Figs. 2 and 3). Not all TMEM16A current was recovered with diC₈-PIP₂ application. 100 μM diC₈-PIP₂ applied without Ca²⁺ had a nominal effect on TMEM16A currents revealing that both Ca²⁺ and PIP₂ are required for the phospholipid to potentiate these channels. We speculate that the incomplete recovery with 100 μM diC₈-PIP₂ applied with Ca²⁺ may reflect a slow rate of PIP₂ integration into the membrane. This is consistent with our observation that the current recovery was diminished when longer acyl chains were applied to patches (Fig. S2). Alternatively, the incomplete recovery may reflect the role of a Ca²⁺-dependent recovery of PIP₂. PIP₂ recovery can be more pronounced at lower Ca²⁺ than at higher Ca²⁺ (17). This complex relationship between PIP₂ and Ca²⁺ needs to be explored further in our system.

Phospholipid-mediated recovery of TMEM16A currents following rundown requires phosphorylation of the inositol ring. Accordingly, we found that 100 μM diC₈-PI, the phospholipid lacking the negatively charged phosphate head groups, was unable to recover TMEM16A currents (Fig. 3). In contrast to the recent report that suggests that the neutral acyl chain of fatty acids is sufficient to regulate TMEM16A (15), our findings suggest that TMEM16A potentiation by PIP₂ requires the presence of phosphate heads which are lacking in lipids like oleic acid or cholesterol. Our results do not preclude the possibility that fatty acids change PIP₂ membrane content, but they do suggest that just a fatty acid tail or backbone like that from PIP₂ is not sufficient for channel potentiation. Consistent with this idea, we observed that adding PI(4)P, the precursor for PIP₂,
containing a singular phosphate at position 4 of the phosphoinositol head group, recovered TMEM16A-conducted Cl⁻ currents (Fig. 3).

Our data suggested that TMEM16A current rundown in the excised patch was caused by PIP₂ depletion. We therefore reasoned that we ought to be able to speed TMEM16A current rundown by scavenging PIP₂ present in the patch. Indeed, application of one of two different PIP₂-scavenging compounds, anti-PIP₂ or neomycin, sped up TMEM16A current rundown (Fig. 4). Other groups have shown that anti-PIP₂ and neomycin effectively scavenged PIP₂ without disrupting ion conductance during single-channel recordings (33) or in excised macropatches expressing the inwardly rectifying K⁺ channels (34, 35).

PIP₂ depletion occurs in excised patches because of the continued activity of membrane-associated phosphatases to dephosphorylate the inositol head group, without the activity of the countering kinases. If TMEM16A current rundown in excised patches is the result of PIP₂ dephosphorylation, we predicted that by enabling kinases to rephosphorylate the lipid or by inhibiting phosphatase activity, we ought to be able to slow current rundown. Indeed, providing patches with Mg-ATP to fuel membrane-anchored kinases to make PIP₂, resulted in TMEM16A currents that ran down significantly more slowly compared with control recordings (Fig. 5). Although it is possible that Mg-ATP could bind to and alter the activity of other proteins in the excised patches, in the context of the other data included in this manuscript, it is likely that Mg-ATP is fueling kinases needed to rephosphorylate PIP₂. In a parallel series of included in this manuscript, it is likely that Mg-ATP is fueling kinases in the excised patches, in the context of the other data including those used during these particular experiments, can associate with PIP₂ (36). Consequently, stearic acids could alter membrane PIP₂ and therefore alter TMEM16A-conducted currents. We sought to resolve these seemingly conflicting results by recording from natively expressed TMEM16A channels in X. laevis oocytes to determine whether PIP₂ regulates TMEM16A gating. Using direct experimental methods, here we demonstrate that TMEM16A requires both membrane PIP₂ and intracellular Ca²⁺ to conduct currents in X. laevis oocytes.

Despite our demonstration that TMEM16A gating is regulated by PIP₂, the exact mechanism involved is yet to be determined. PIP₂ regulates other ion channels by diverse mechanisms (19). For example, PIP₂ may regulate TMEM16A currents by acting on an accessory protein, exemplified by PIP₂ binding to KCNE1 to regulate currents conducted by the voltage-gated potassium channel KCNQ1 (37). Alternatively, PIP₂ can form electrostatic interactions between a cluster of positive charges on a channel and the negative charges on the phosphoinositol head of PIP₂ as has been observed for transient receptor potential vanilloid 5 (TRPV5) (38), inward-rectifier K⁺ (Kir2.2) (39), and G protein–gated K⁺ (GIRK2) (40). Unlike KCNQ1, no accessory protein has been revealed for TMEM16A. Although CLCA1 has been identified as an accessory protein for TMEM16A channels (41), neither this protein nor the RNA are found in mature X. laevis eggs or the fertilization-incompetent oocytes (42–44). Our data, however, do not preclude an indirect mechanism for PIP₂ regulation of TMEM16A by another accessory protein for TMEM16A. We speculate that TMEM16A and PIP₂ may form electrostatic interactions similar to those revealed by the TRPV5, Kir2.2, and GIRK2 PIP₂-binding structures.

Electrostatic interactions mediate PIP₂ binding to the closely related cation channel, TMEM16F (45). The putative PIP₂-binding site in TMEM16F is comprised of positively charged residues, and neutralizing these residues perturbed PIP₂’s ability to potentiate TMEM16F currents (45). It is possible that a conserved domain mediates PIP₂ interactions with all TMEM16 family proteins including TMEM16A channels. Although the binding domain may be shared, the effects on proteins will most certainly differ. For example, the Ca²⁺-activated Cl⁻ channel TMEM16B is inhibited by PIP₂ (17). Yet, a shared PIP₂ regulation among TMEM16 family proteins is perhaps not surprising given that this protein family includes several lipid scrambles whose physiologic function requires their interaction with charged lipids.

A requirement for PIP₂ in TMEM16A activation is intriguing because the PIP₂ cleavage into IP₃ to signal Ca²⁺ release from the ER would seemingly oppose the ability of increased Ca²⁺ to activate the channel. Yet several independent studies have revealed that an IP₃-evoked Ca²⁺ release from the ER opens...
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TMEM16A channels to signal diverse physiologic processes including signaling contraction of lymphatic vessels (46, 47) to activation of the fast polyspermy block (43, 48). We propose that in systems that use an IP$_3$ pathway to activate TMEM16A channels, the amount of PIP$_2$ cleaved to generate IP$_3$ matters greatly. For example, stimuli that evoke the cleavage of only a moderate amount of PIP$_2$ to increase IP$_3$ and evoke a Ca$^{2+}$ release from the ER will ultimately signal the opening of more TMEM16A channels compared with other stimuli that signal the cleavage of most of the membrane PIP$_2$ to increase IP$_3$ and evoke an even larger Ca$^{2+}$ release from the ER. Perhaps TMEM16A discriminates its own Ca$^{2+}$ signal from Ca$^{2+}$-signaling cascades that exclude it by seeking interactions with PIP$_2$ at the plasma membrane. Moreover, these studies reveal that the physiologic mechanisms underlying TMEM16A opening are more complicated than simply the presence or absence of intracellular Ca$^{2+}$, thereby enabling TMEM16A to play diverse roles in different cell types.

Understanding how the channel is regulated lays the conceptual framework for drugging this novel interaction in disease. In hypertension, the overconstriction of vessels can be alleviated by inhibiting Ca$^{2+}$-activated Cl$^{-}$ channels like TMEM16A (49). In cystic fibrosis, a condition arising from a dysfunctional Cl$^{-}$ channel, increasing TMEM16A activity could rescue the defects caused by poor Cl$^{-}$ transport (50). By understanding the interaction between PIP$_2$ and TMEM16A, drugs targeting the PIP$_2$-TMEM16A interaction site can be designed to either inhibit TMEM16A activity with the benefit of lowering blood pressure or increase its activity to promote Cl$^{-}$ transport.

**Experimental procedures**

**Reagents**

diC8-PIP$_2$, diC8-PI, diC8-PI(4)P, PIP$_2$ 18:0/20:4, and anti-PIP$_2$ IgM (catalog no. Z-P045, lot no. 080416) were obtained from Echelon Biosciences. MgCl$_2$ was obtained from Sigma. Unless otherwise noted, all other reagents were purchased from Thermo Fisher Scientific.

**Solutions**

All inside-out patch clamp recordings were made in HEPES-buffered saline solution (in mM): 130 NaCl and 3 HEPES, pH 7.2, and filtered using a sterile, 0.2-μm polystyrene filter. For Ca$^{2+}$-free recordings, this solution was supplemented with 0.2 μM EGTA as indicated. For solutions used during Ca$^{2+}$ application, the HEPES-buffered saline solution was supplemented with 2 mM CaCl$_2$ and with indicated reagents. For current recovery experiments, one of the diC8 analogs or natural PIP$_2$ was added to the HEPES-buffered saline solution supplemented with 2 mM CaCl$_2$. Natural PIP$_2$ was dissolved and sonicated prior to use because of its longer acyl chain.

Oocyte wash and storage solution were made as follows: Oocyte Ringers 2 (in mM): 82.5 NaCl, 2.5 KCl, 1 MgCl$_2$, and 5 mM HEPES, pH 7.2. ND96 (in mM): supplemented with 5 sodium pyruvate and 100 mg/liter gentamycin, pH 7.6, and filtered with a sterile, 0.2-μm polystyrene filter.

**Animals**

Animal procedures were conducted using accepted standards of humane animal care and approved by the Animal Care and Use Committee at the University of Pittsburgh. *X. laevis* adult, oocyte-positive females were obtained commercially (RRID:NXR_0031, NASCO, Fort Atkinson, WI) and housed at 18 °C with 12/12-h light/dark cycle.

**Oocyte collection**

Oocytes were collected from *X. laevis* females anesthetized by immersion in 1.0 g/liter tricaine, pH 7.4, for 30 min. Ovarian sacs containing the oocytes were removed from the female, manually pulled apart, and incubated for 90 min in 1 mg/ml collagenase diluted in the ND96 solution. Collagenase was removed by several Oocyte Ringers 2 rinses, and healthy oocytes were stored at 14 °C in ND96 for up to 14 days.

**Patch clamp recordings**

Patch clamp recordings were made on *X. laevis* oocytes following the manual removal of the vitelline membrane. Current recordings were made in the inside-out configuration of the patch clamp technique (51) with an EPC-10 USB patch clamp amplifier (HEKA Elektronik). Briefly, after formation of a gigaseal (greater than 1 gigahm), inside-out patches were excised in HEPES-buffered saline solution lacking EGTA (resistances often decreased to 20–200 megohm in solutions lacking EGTA but returned to greater than 1 gigahm with EGTA application). Data were collected at a rate of 10 kHz. Glass pipettes were pulled from borosilicate glass (outer diameter 1.5 mm, inner diameter 0.86 mm; Warner Instruments), fire polished (Narshige microforge), and had a resistance of 0.4–1.5 megohm. Lipids were applied to excised inside-out patches in a RC-28 chamber (Warner Instruments). All other solutions were applied using a VC-8 fast perfusion system (Warner Instruments). Experiments were initiated within 10 s of patch excision.

**Data analysis**

Patch clamp data were acquired with PATCHMASTER (HEKA Elektronik) and analyzed with Igor Pro (RRID:SCR_000325, WaveMetrics), Patchers Power Tools (RRID:SCR_001950), and Excel (RRID:SCR_016137, Microsoft). Currents were normalized such that the basal currents recorded in Ca$^{2+}$-free conditions were equated to 0 and the peak currents obtained with 2 mM intracellular Ca$^{2+}$ were normalized to 1.

Data from various experimental conditions are displayed in Tukey box plot distributions where the central line represents the median value, the box depicts 25–75% of the data range, and the whiskers span 10–90%.

To facilitate comparison between the kinetics of current rundown recorded under experimental conditions to controls, the normalized current for each condition is plotted together with the averaged rundown for associated control, either the 2 mM Ca$^{2+}$ or the 1.5 mM Mg-AMP. These controls are plotted with dashed lines, and represent an idealized averaged normalized current versus time calculated using the single exponential Equation 1:
where $Y(x)$, $Y_0$, $x$, and $\tau$ represent the current at time $x$, initial current, time, and rate of current rundown, respectively. Briefly, traces were collected under either condition and fitted with the single exponential equation above to derive the $Y_0$, $x$, and $\tau$. These values where collected for each individual plot. To create averaged plots of current rundown for control and Mg-AMP conditions, single exponential functions using the averaged variables were plotted along with the normalized current versus time graphs. The predicted current traces were then plotted as the dashed lines.

To compare the magnitude of current recovered using the synthetic lipid analogs (diC8-PIP2, diC8-PI, diC8-PI(4)P) and natural PIP2, the fold change in current recovered was calculated by dividing the peak current after diC8-PIP2, diC8-PI, diC8-PI(4)P or natural PIP2 addition by baseline current. The peak current was defined as the highest current obtained after diC8-PIP2, diC8-PI, diC8-PI(4)P, or natural PIP2 addition. The baseline current was equated to 1 and defined as the current observed at point of diC8-PIP2 or diC8-PI addition.

All experimental conditions include trials that were conducted on multiple days with oocytes collected from different females. Two-tailed analyses of variance (ANOVAs) were used to report differences between experiments conditions, followed by post hoc $t$ tests to discern differences between particular experimental treatments.

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