Phosphate position is key in mediating transmembrane ion channel TMEM16A–phosphatidylinositol 4,5-bisphosphate interaction

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TransMEMbrane 16A (TMEM16A) is a Ca2+-activated Cl− channel that plays critical roles in regulating diverse physiologic processes, including vascular tone, sensory signal transduction, and mucosal secretion. In addition to Ca2+, TMEM16A activation requires the membrane lipid phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2). However, the structural determinants mediating this interaction are not clear. Here, we interrogated the parts of the PI(4,5)P2 head group that mediate its interaction with TMEM16A by using patch- and two-electrode voltage-clamp recordings on oocytes from the African clawed frog Xenopus laevis, which endogenously express TMEM16A channels. During continuous application of Ca2+ to excised inside-out patches, we found that TMEM16A-conducted currents decayed shortly after patch excision. Following this rundown, we show that the application of a synthetic PI(4,5)P2 analog produced current recovery. Furthermore, inducible dephosphorylation of PI(4,5)P2, reduces TMEM16A-conducted currents. Application of PI(4,5)P2 analogs with different phosphate orientations yielded distinct amounts of current recovery, and only lipids that include a phosphate at the 4′ position effectively recovered TMEM16A currents. Taken together, these findings improve our understanding of how PI(4,5)P2 binds to and potentiates TMEM16A channels.

The broadly expressed Ca2+-activated Cl− channel TransMEMbrane 16A (TMEM16A) regulates diverse physiologic processes, including contraction of arterial smooth muscle (1–3), transduction of sensory signals (4, 5), mucosal secretion (6, 7), peristalsis of the gastrointestinal tract (8, 9), and fertilization (10, 11). TMEM16A dysregulation is associated with diseases, such as hypertension (1, 12) and cancers of various tissues (e.g., the breast (13) and pancreas (14)). Owing to its original identification as a cancer biomarker, TMEM16A is also known as Discovered On Gastrointestinal Stromal Tumors protein 1 (DOG1), Oral Cancer Overexpressed 2 (ORAOV2), and Tumor Amplified and Overexpressed 2 (TAOS-2).

Since the original characterization of TMEM16A as a Cl− conducting channel in 2008 (15–17), substantial progress has been made in determining its structural and biophysical properties. TMEM16A channels are homodimers with each independently operating subunit comprising 10 transmembrane domains with large intracellular N and C termini (18, 19). Transmembrane domains 3 to 7 comprise the Cl− conducting pore (20, 21), and five conserved acidic amino acids from transmembrane domains 6 to 8 coordinate two Ca2+ ions (18, 22, 23). The channel gates differently depend on whether one or two Ca2+ ions occupy the binding site (24). The Ca2+-binding site is embedded in the plasma membrane electric field, giving rise to the weak voltage dependence of TMEM16A gating at intracellular Ca2+ concentrations below saturation (18, 22, 23).

In addition to intracellular Ca2+, TMEM16A also requires the acidic phospholipid phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) in order to transition to the open conformation (25–29). Like the currents of other channels potentiated by PI(4,5)P2 (30), TMEM16A currents recorded using the inside–out configuration of the patch-clamp technique rundown over time, even with continued application of intracellular Ca2+. For many channels, a few basic amino acids at the plasma membrane–cytoplasm interface enable electrostatic interactions with PI(4,5)P2, which differs from other highly structured PI(4,5)P2-binding sites (e.g., the pleckstrin homology domain, a common structural motif containing more than 100 amino acids (30)). Results from several groups suggest that TMEM16A–PI(4,5)P2 binding is electrostatic and relies on the negative charge carried by the PI(4,5)P2 phosphates. Indeed, neutralization of six positively charged arginine and lysine residues clustered near the cytoplasmic interface of transmembrane domains 3 to 5 was shown to speed TMEM16A desensitization (26). Another group identified three discrete TMEM16A PI(4,5)P2-binding sites that mediate interactions with phosphate groups as well as the membrane-embedded fatty acid tail (29).

Here, we sought to uncover the molecular determinants of PI(4,5)P2 interactions with TMEM16A channels. We made electrophysiology recordings of African clawed frog (Xenopus laevis) oocytes, which endogenously and abundantly express TMEM16A channels (16). Using a combination of excised inside–out patch clamp along with two-electrode voltage-
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clamp (TEVC) recordings, we found that TMEM16A channels are regulated by PI(4,5)P2 in both the excised patch and whole cell. In excised patches, TMEM16A-conducted currents rundown following patch excision and can be recovered with the application of water-soluble PI(4,5)P2 analogs. The extent of recovery depended on the phosphate positions on the inositol ring, revealing a prominent role for the phosphate at the 4′ position. Moreover, we found that removing the 5′ phosphate with a voltage-sensing phosphatase (VSP) did not significantly speed rundown. Together, these findings establish that the inositol-ring phosphate at the 4′ position is key for PI(4,5)P2 regulation of TMEM16A channel activity.

Results

TMEM16A currents recorded from excised inside–out patches rundown and are recovered by PI(4,5)P2

Endogenous TMEM16A-conducted currents were recorded from *X. laevis* oocytes in the excised inside–out patch configuration. Briefly, inside–out patch currents were recorded during 150 ms steps to −60 and +60 mV before and during application of saturating Ca2+. As previously reported, we observed robust Ca2+-activated TMEM16A-conducted currents shortly after excision (28). Figure 1A depicts currents recorded at −60 and +60 mV at indicated time points (10–180 s) following 2 mM Ca2+ addition. These currents decayed over time despite the continued Ca2+ application (Fig. 1). By fitting single exponential functions (Equation 1) to plots of normalized currents recorded during steps to −60 mV versus time, we found that the average rate of rundown was 80.6 ± 14.8 s (N = 11).

The currents of PI(4,5)P2-regulated channels characteristically rundown when recorded using the inside–out configuration of the patch-clamp technique. Indeed, we found that application of 100 μM of the soluble dioctanoyl phosphatidylinositol 4,5-bisphosphate (diC8-PI(4,5)P2) recovered TMEM16A currents (28). Figure 1D shows an example plot of TMEM16A-conducted currents recorded at −60 mV versus time, and before and during application of 100 μM diC8-PI(4,5)P2. In nine independent trials, we observed that diC8-PI(4,5)P2 recovered an average of 3.5 ± 0.4-fold current (Fig. 1E).

To explore whether reducing membrane PI(4,5)P2 also depletes TMEM16A currents in whole oocytes, we employed a rapamycin-induced dimerization system to translocate a cytosolic phosphatase, pseudojanin, to a membrane-anchored domain Lyn11 (31) (Fig 2A). At the plasma membrane, the synthetic enzyme pseudojanin dephosphorylates PI(4,5)P2 at both the 4′ and 5′ positions (31). Using TEVC recordings, we compared how rapamycin application altered the TMEM16A-conducting currents in wildtype *X. laevis* oocytes with those expressing pseudojanin and Lyn11-mCherry. UV light application photoactivated caged-IP3 to increase intracellular Ca2+ in oocytes clamped at −80 mV. In wildtype oocytes, rapamycin application did not alter the TMEM16A-conducted currents; we observed an average of 101 ± 5.8% with rapamycin treatment compared with currents observed before treatment (N = 13) (Fig. 2, B and C). By contrast, we observed that in cells expressing Lyn11 and pseudojanin, the percent of remaining current after rapamycin-induced dimerization was an average of 80.8 ± 6.1% (N = 19, p = 0.02, t test) (Fig. 2, B and D). Together, these data demonstrate that PI(4,5)P2 potentiates the endogenous TMEM16A channels in *X. laevis* oocytes in both excised patches and whole cells.

Phospholipids differentially recovered TMEM16A currents following rundown

To further characterize the TMEM16A–PI(4,5)P2 interaction, we explored whether the number of negative charges carried by the inositol phosphate altered the ability of the lipid to regulate channel activity. We began by testing the hypothesis that applying phosphoinositol analogs with more phosphate groups, and therefore more negative charges, would be more effective at recovering TMEM16A current following rundown. We applied a water-soluble analog of dioctanoyl phosphatidylinositol 3,4,5-trisphosphate (diC8-PIP3) to excised inside–out patches after current had steadily rundown. In seven independent trials, we observed that 100 μM diC8-PIP3 application recovered an average of 4.6 ± 1.0-fold current, which was not significantly different from the
current recovered by diC8-PI(4,5)P₂ (Fig. 3A) \( (p = 0.27, \) Tukey’s honestly significant difference [HSD] test). Figure 3B shows an example plot of TMEM16A-conducted currents \textit{versus} time before and during diC8-PIP₃ application. These data reveal that the phosphate groups’ negative charge is insufficient to predict how the phospholipid analogs potentiate TMEM16A currents.

We next examined whether the relative phosphate position determined how effective a phospholipid recovered TMEM16A. We applied diC8-phospholipids that included two variably placed phosphate groups on the inositol ring. We started with another vicinal diphospholipid: dioctanoyl phosphatidylinositol 3,4-bisphosphate (diC8-PI(3,4)P₂) (Fig. 3C). In six independent trials, we observed that 100 \( \mu \)M of diC8-PI(3,4)P₂ recovered an average of 3.1 ± 0.7-fold current \( (\text{Fig. 3, A and C}) \), which was similar to the recovery observed with diC8-PI(4,5)P₂ \( (3.5 \pm 0.4\text{-fold current}, N = 8) \).

Not all biphasate analogs effectively recovered TMEM16A-conducted current. In five independent trials, dioctanoyl phosphatidylinositol 3,5-bisphosphate (diC8-PI(3,5)P₂) recovered only 1.3 ± 0.1-fold current \( (\text{Fig. 3, A and D}) \), significantly less than that recovered by diC8-PI(4,5)P₂ \( (p < 0.01, \) Tukey’s HSD test). These data reveal that the number of negatively charged phosphate groups does not explain how effectively a phospholipid can recover TMEM16A-conducted current. The two phospholipids that effectively regulated TMEM16A currents shared two characteristics: they both had vicinal phosphate groups and phosphate groups at the 4’ position.

\textbf{Phosphate at position 4’ of the inositol ring is key in the interaction of PI(4,5)P₂ with TMEM16A.}

We predicted that if vicinal phosphates were required to potentiate TMEM16A, then diC8 analogs with a single phospholipid should not recover TMEM16A currents following rundown. By contrast, if a phosphate at the 4’ position is required to potentiate TMEM16A-conducted current, only...
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dioctanoyl 4-monophosphate (diC8-PI4P), but not dioctanoyl 3-monophosphate (diC8-PI3P) or dioctanoyl 5-monophosphate (diC8-PI5P), should recover TMEM16A currents. To discriminate between these possibilities, we applied phosphoinositid monophosphates to excised patches following rundown. We first assessed if phosphates at positions 3’ and 5’ of the inositol head would also recover TMEM16A following rundown. Figure 4, A and B shows example plots of TMEM16A-conducted currents recorded at −60 mV versus time, before and during diC8-PI3P or diC8-PI5P application. We observed that the application of 100 μM diC8-PI3P recovered 1.5 ± 0.3-fold current (N = 5) and that diC8-PI5P only recovered 1.3 ± 0.1-fold current (Fig. 4, A–C).

Next, we assessed if the phosphate at position 4’ was indeed important for phosphoinositid potentiation of TMEM16A-conducted Cl− currents. We applied diC8-PI4P to excised inside–out patches after the current had stably rundown. We recorded TMEM16A-conducted currents at −60 mV before and during the application of 100 μM diC8-PI4P. In seven separate trials, we observed that 100 μM diC8-PI4P application recovered 2.4 ± 0.9-fold current (Fig. 4, C and D). The currents recovered by diC8-PI4P were similar albeit smaller than the 3.5 ± 0.4-fold recovery observed with diC8-PI(4,5)P2 (p > 0.1, Tukey’s HSD test) (Fig. 4C). Together, these data were consistent with the hypothesis that a phosphate group at the 4’ position was critical for the phospholipid to recover TMEM16A-conducted currents.

As another test of the importance of a 4’ phosphate, we exogenously expressed a VSP in X. laevis oocytes and used membrane depolarization to activate dephosphorylation of PI(4,5)P2 at the 5’ position in excised patches (Fig. 5A). If the 4’ phosphate is required for the phospholipid to potentiate TMEM16A channels, then activation of VSP should not speed rundown. By contrast, if vicinal phosphates are required for the lipid to regulate these currents, then VSP activation should speed rundown. We used the VSP endogenously expressed in X. laevis sperm (XI-VSP) with a GFP on the N terminus. GFP fluorescence confirmed VSP expression (Fig. 5B) prior to patch clamp recordings. Notably, the XI-VSP enzyme is turned on by voltages more positive than −20 mV, with maximal activation observed at +40 mV (32). We focused on oocytes with GFP fluorescence and recorded TMEM16A Ca2+–evoked Cl− currents. In five independent trials, we recorded TMEM16A-conducted currents at −60 mV before and during the application of Ca2+ in patches while activating XI-VSP at depolarizing potentials (+60 mV). We observed that the rate of current rundown.

Figure 4. Phospholipids with phosphates at position 4’ of the inositol ring recover current. Soluble synthetic analogs of PI3P, PI4P, and PI5P were applied to excised inside–out patches once current had stably rundown. Currents were recorded at −60 mV. Representative plots of normalized currents versus time, before and during application of 100 μM diC8-PI3P (A), 100 μM diC8-PI5P (B), or 100 μM diC8-PI4P (D). C, box plot distribution of the fold current recovered after the application of diC8-PI(4,5)P2 (N = 9), diC8-PI3P (N = 7), diC8-PI4P (N = 7), or diC8-PI5P (N = 5). * denotes p < 0.05 between indicated treatment and diC8-PI(4,5)P2 as determined by ANOVA and Tukey’s HSD post hoc tests. diC8-PI3P, dioctanoyl 3-monophosphate; diC8-PI4P, dioctanoyl 4-monophosphate; diC8-PI5P, dioctanoyl 5-monophosphate; HSD, honestly significant difference.

Figure 5. XI-VSP does not significantly change TMEM16A current rundown. Inside–out patch-clamp recordings were conducted on macro-patches excised from Xenopus laevis oocytes expressing XI-VSP. A, schematic depicting a VSP tagged with GFP, B, confocal and bright-field images of a representative X. laevis oocyte expressing GFP-tagged XI-VSP at the plasma membrane. Bar denotes 200 μm. C, box plot distribution of the rate of current decay (τ), measured by fitting plots of relative current versus time with single exponentials for the XI-VSP expressing (purple) (N = 6). Background gray dashed lines denote 25 to 75% of the data spread, and the solid line represents the median rate of rundown measured from patches recorded under the control conditions (plotted in Fig. 1C). D, representative plot of normalized currents versus time following VSP activation. TMEM16A, TransMEMbrane 16A; XI-VSP, Xenopus laevis voltage-sensing phosphatase.
rundown in patches expressing XI-VSP at the membrane was 76.1 ± 18.1 s and was not significantly different compared with the control-current rundown (80.5 ± 14.8 s; N = 11; Fig. 5C, t test, p = 0.63). Figure 5D shows an example plot of TMEM16A-conducted currents recorded at −60 mV versus time with XI-VSP activation. The rate of TMEM16A-current rundown is similar in the presence and absence of VSP, consistent with the hypothesis that the 4′ phosphate is critical for phospholipid regulation of TMEM16A-channel activity.

Discussion

This study sought to uncover how PI(4,5)P$_2$ interacts with TMEM16A channels. As we have reported previously (28), we again found that Ca$^{2+}$-activated TMEM16A-conducted currents rundown over time when recorded in the inside−out configuration of the patch-clamp technique, despite the continued application of Ca$^{2+}$. We previously reported that TMEM16A-conducted currents rundown in excised patches because of continued activity of membrane-associated phosphatases that dephosphorylate the inositol head group of PI(4,5)P$_2$, without the activity of counteracting kinases (28). We report that application of the water-soluble phospholipid diC8-PI(4,5)P$_2$ recovered these currents. Here, we in addition report that degradation of PI(4,5)P$_2$ in whole X. laevis oocytes also reduced TMEM16A-conducted currents. By exogenously expressing the soluble phosphatase pseudojanin and the membrane-tethered N terminus of Lyn kinase (Lyn11), we observed a rapamycin-evoked reduction in the membrane-tethered N terminus of Lyn kinase (Lyn11), we expressing the soluble phosphatase pseudojanin and the also reduced TMEM16A-conducted currents. By exogenously expressed Xl-VSP should not significantly alter rundown. We did not observe a significant difference between the rate of rundown in wildtype and XI-VSP–expressing patches (Fig. 5), thereby supporting our hypothesis that the 4′ phosphate is critical for PI(4,5)P$_2$ regulation of TMEM16A-conducted currents.

Our finding that PI(4)P is sufficient to potentiate TMEM16A is surprising given that previous studies in mouse TMEM16A have demonstrated that the 4′ phosphate is dispensable (26). Still others have found that VSP activation does successfully diminish TMEM16A-conducted currents (35).

We attempted to resolve these conflicting findings by performing computer docking of PI(4,5)P$_2$ into a homology model of X. laevis TMEM16A (Fig. 6A). The modeled region (residues 113−931) of X. laevis TMEM16A is 82.2% identical to the corresponding region in the mouse channel and aligns to mouse TMEM16A with an RMSD of 1.1 Å (Fig. 5A). Notably, our model preserves putative PI(4,5)P$_2$-binding sites identified in mouse TMEM16A (26, 29) including a strongly basic cluster formed between TM5 and the TM2–3 linker. Docking diC8-PI(4,5)P$_2$ into the X. laevis TMEM16A homology model suggests that the 4′ phosphate forms a salt bridge with the conserved residue K446 (K451 in the mouse TMEM16A) (Fig. 6B). Notably, K446 is part of the EAV K motif also known as the c-segment (exon 13) of mouse TMEM16A. This motif is removed in a splice variant (EAVK, A448−451, in the linker connecting transmembrane domains 2 and 3) of the mouse channel (36). The ΔEAVK splice variant was used in a previous study reporting that PI4P did not potentiate TMEM16A-conducted currents (26). This difference is notable because the hypothesized PI(4,5)P$_2$-binding sites are otherwise nearly
The required 4′ phosphate may support a previously proposed networked PI(4,5)P_2-binding model for mouse TMEM16A (29). In this model, TMEM16A contains three PI(4,5)P_2-binding sites, formed by basic residues that, when neutralized, reduce PI(4,5)P_2 sensitivity (29). These sites are conserved in X. laevis, and the 4′ phosphate may be required for binding one or more of these sites. However, this model does not account for the apparent contribution of mouse TMEM16A residue K451 in binding PI(4,5)P_2, as the proposed PI(4,5)P_2 sites are located elsewhere (29, 35, 39). One possibility is that our proposed binding site (Fig. 6B) works to allosterically regulate the activation of TMEM16A. Further study is needed to resolve this intriguing hypothesis.

### Experimental procedures

#### Reagents

The following dioctanoyl phospholipids were obtained from Echelon Biosciences: diC8–PI3P, diC8–PI4P, diC8–PI5P, diC8–PI(3,4)P_2, diC8–PI(4,5)P_2, diC8–PI(3,5)P_2, and diC8–PIP_3. Plasmids encoding Lyn11-targeted FRB and pseudojanin were purchased from Addgene (40). Unless otherwise noted, all other reagents were purchased from Thermo Fisher Scientific.

#### Animals

Animal procedures used were approved by the Animal Care and Use Committee at the University of Pittsburgh and were consistent with the accepted standards of humane animal care. X. laevis adult oocyte-positive females were obtained commercially (Nasco) and housed at 18 °C with a 12/12-h light/dark cycle.

#### Oocyte collection

X. laevis females were anesthetized by immersion in 1.0 g/l tricaine (pH 7.4) for 30 min before oocytes were collected. The ovarian sacs were surgically obtained and manually pulled apart using blunt forceps. Oocytes were treated with a 90-min incubation in 1 mg/ml collagenase in the ND96 solution and then repeatedly rinsed in oocyte Ringer’s solution 2 (OR2) to remove collagenase. Healthy oocytes were stored at 14 °C in pyruvate- and gentamycin-supplemented ND96 solution.

#### Solutions

Inside–out patch-clamp recordings were conducted in a Hepes-buffered saline solution made as follows (in millimolar): 130 NaCl and 3 Hepes, pH 7.2, and filtered using a sterile 0.2-μm polystyrene filter (28). The Hepes-buffered saline solution was supplemented with 0.2 μM EGTA for Ca^{2+}-free recordings. For recordings made with intracellular Ca^{2+}, the Hepes-buffered saline solution was supplemented with 2 mM CaCl_2 with indicated reagents. For current recovery experiments, the diC8 analogs were added to the Ca^{2+}-containing Hepes-buffered saline solution and applied as indicated. TEVC recordings were conducted in solution ND96 made as follows (in millimolar): 96 NaCl, 2 KCl, 1 MgCl_2, 10 Hepes, pH 7.6. The solution was filtered with a sterile 0.2-μm polystyrene filter (16).
The oocyte wash solution, called OR2, and storage solution, ND96, were made as follows. OR2 (in millimolar): 82.5 NaCl, 2.5 KCl, 1 MgCl₂, and 5 mM Hepes, pH 7.2. ND96 (in millimolar): OR2 supplemented with 5 mM sodium pyruvate, 100 mg/l gentamycin, pH 7.6, and 0.2 μm polystyrene filtered.

**Patch-clamp recordings**

Patch-clamp recordings were made on *X. laevis* oocytes after manually removing the vitelline membrane. TMEM16A current recordings made in the inside-out configuration of the patch-clamp technique (41) used an EPC-10 USB patch-clamp amplifier (HEKA Elektronik). Data were acquired with Patchmaster software (HEKA Elektronik). Briefly, upon formation of a gigaseal (greater than 1 GΩ), inside-out patches were excised in Hepes-buffered saline solution lacking both EGTA and added CaCl₂. In this EGTA-free Hepes-buffered saline, the patch resistances often decreased to 20 to 200 MΩ following excision but returned to greater than 1 GΩ 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) and fire polished (Narishige Microforge). Each pipette had a resistance of 0.4 to 1.5 MΩ. All data collection for inside-out patch experiments was initiated within 10 s of patch excision. All diC₈ lipid analogs were applied to excised inside-out patches in an RC-28 chamber (Warner Instruments). Other solutions were applied to excised patches using a VC-8 fast perfusion system (Warner Instruments).

Patch-clamp data were analyzed with Excel (Microsoft) and IGOR Pro (Wavemetrics) with Patchers Power Tools. Currents were processed such that peak currents obtained with 2 mM intracellular Ca²⁺ application were normalized to 1. The basal currents recorded once the patch reached a steady state following rundown.

To calculate the rate of rundown, plots of normalized current (%). The equation used was:

\[ Y(x) = Y_0 e^{-\frac{x}{\tau}} \]  (1)

where \( Y_0 \), \( x \), \( \tau \), and \( Y(x) \) represent the initial current, time, rate of current rundown, and current at time \( x \) (28).

To compare the current recovered following application of different synthetic lipid analogs, the fold change in current recovered was calculated by dividing the peak current after diC₈-analog addition by the baseline current. The peak current was defined as the highest current obtained after diC₈-analog addition. The baseline current was defined as the current observed at the point of diC₈-analog addition. The equation used was:

\[ \text{Fold recovery} = \frac{\text{Maximum current after analog addition}}{\text{Baseline current at analog addition}} \]  (2)

A fold recovery of one relates to unchanged current, and a recovery >1 indicates that diC₈-analog application increased the current.

Each experimental condition includes trials conducted on multiple days with oocytes collected from different females.

**Exogenous protein expression in X. laevis oocytes**

The complementary DNAs encoding the Lyn₁₁ and pseudojanin constructs were engineered into the GEMHE vector using overlapping extension PCR. The sequences for all constructs were verified by automated Sanger sequencing (Geneviz). The XI-VSP construct was provided by L. Jaffe (University of Connecticut (32)). The cRNAs for these constructs were transcribed using the T7 mMessage mMachine Ultra kit (Ambion). Defolliculated oocytes were injected with 5 ng of cRNA for both Lyn₁₁ and pseudojanin or XI-VSP. Injected oocytes were screened for Lyn₁₁-mCherry or XI-VSP-GFP expression at the membrane using confocal imaging 48 h after cRNA injection.

**TEVC recordings**

TEVC recordings were made using TEV-200A amplifiers (Dagan Co) and digitized by Axon Digidata 1550A (Molecular Devices). Data were acquired with pClamp Software (Molecular Devices) at a rate of 5 kHz. Recordings were made on *X. laevis* oocytes 48 h after cRNA injection and 1 to 4 h after injection of photolabile IP₃ analog myoinositol 1,4,5-trisphosphate, P4(5)-1-(2-nitrophenyl) ethyl ester (caged IP₃). IP₃-evoked currents were recorded in the TEVC configuration at ~80 mV from *X. laevis* oocytes. Oocytes were injected with a 200 μM caged IP₃ stock made in double-distilled water to reach a final concentration of 5 μM within the oocyte and incubated in the dark at 18 °C before recording (10).

10 Pipettes of 1 to 8 MΩ resistance were pulled from borosilicate glass and filled with 1 M KCl. A 250-ms exposure to UV light (Ultra High Power White LED Illuminator, 380-603; Prizmatix) was used to release the nitrophenyl cage on IP₃. A liquid light source guided the light to oocytes in the recording chambers (RC-26G; Warner Instruments). The solution bathing the oocyte was changed with the gravity-fed pinch valve VC-8 solution changer (Warner Instruments). It is impossible to directly compare current amplitudes generated in different oocytes because of the innate variability of the experimental setup (e.g., positioning of the UV light or the exact amount of caged IP₃ in each oocyte). Changes in the background-subtracted peak currents were quantified from two consecutive recordings. The difference between the peaks of these consecutive recordings was used to calculate the remaining current (%).

To compare the effects of rapamycin on TMEM16A currents in wildtype oocytes, or oocytes expressing pseudojanin and Lyn₁₁, we first normalized the currents observed by subtracting baseline currents from peak currents for both currents measured. Then, we calculated the percent change in current after rapamycin treatment relative to before treatment. The percent change was plotted as the proportion of remaining current observed with rapamycin.

We sought to express a channel whose currents would diminish with VSP as a positive control for the XI-VSP
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experiments. VSP activation reportedly diminished currents from Kir2.1 channel (42). Here, we expressed Kir2.1 in X. laevis oocytes (Addgene plasmids 32669 and 32641, (43, 44)) and used TEVC to record whole-cell currents while ramping the voltage from −150 to +50 mV, over 2 s. Recording in a potassium-based solution comprised of (in millimolar): 90 KCl, 5 Hepes, 0.1 CaCl2, 1 MgCl2, pH 7.6 (45). We did not observe rectification from either channel.

Quantification and statistical analyses
Data for each experimental condition are reported as mean ± SEM values and displayed in Tukey’s box plot distributions. Tukey’s box plot displays the data between 25 and 75 percentile, and the whiskers span 10 to 90. Phosphoinositide distributions. Tukey ramping the voltage from 44)) and used TEVC to record whole-cell currents while methionines to methionines, using the default parameters, except we changed all selenomethionines through Schrödinger Maestro electron microscopy at 3.30 Å. We ran the 7B5D structure identity with Quanti X. laevis oocytes (Addgene plasmids 32669 and 32641, (43, 44)) and used TEVC to record whole-cell currents while ramping the voltage from −150 to +50 mV, over 2 s. Recording in a potassium-based solution comprised of (in millimolar): 90 KCl, 5 Hepes, 0.1 CaCl2, 1 MgCl2, pH 7.6 (45). We did not observe rectification from either channel.

Quantification and statistical analyses
Data for each experimental condition are reported as mean ± SEM values and displayed in Tukey’s box plot distributions. Tukey’s box plot displays the data between 25 and 75 percentile, and the whiskers span 10 to 90. Phosphoinositide current recovery was analyzed using ANOVAs followed by post hoc Tukey’s HSD tests to compare each phosphoinositide to diC8-PI(4,5)P2. Two-tailed t tests were used to determine differences between XI-VSP and control patch-clamp recordings and pseudojanin currents in rapamycin versus wild-type oocytes treated with rapamycin for TEVC recordings.

Homology modeling
To identify a suitable template for homology modeling, we performed a BLAST search of the Protein Data Bank (46) using the UniProt B5SVV6 sequence (X. laevis TMEM16A) (47). The search identified a dimeric structure of Mus musculus TMEM16A (Protein Data Bank ID: 7B5D (48); 76.9% identity with X. laevis TMEM16A), resolved using cryo-electron microscopy at 3.30 Å. We ran the 7B5D structure through Schrödinger Maestro’s Protein Preparation Wizard using the default parameters, except we changed all selenomethionines to methionines, filled in any missing side chains, deleted all water molecules, and did not perform a final restrained minimization.

We then used the Maestro Homology Modeling module to generate a homology model of X. laevis TMEM16A based on the prepared 7B5D template. We performed knowledge-based modeling using the multiple templates (homomultimer) option to build a dimeric (rather than monomeric) model. We ran the resulting homology model through the Protein Preparation Wizard. We again used the default parameters, except we capped the terminal ends, changed all selenomethionines to methionines, filled in any missing side chains, and deleted all water molecules. We also performed a final restrained minimization.

Docking
We used Schrödinger Maestro to prepare receptor grids for docking (default parameters). Each docking grid was centered on residues K446, K592, and R450. Given that TMEM16A is a homodimer, there are two such sites. We prepared separate receptor grids for each site.

To prepare small-molecule models of diC8-PI(4,5)P2 and IP3 for docking, we first obtained the SMILES strings of these two compounds from PubChem (49, 50). We then used Maestro’s LigPrep module (default parameters) to generate multiple compound models, as required to account for alternative protonation states. LigPrep produced 11 distinct ligand models: four models of PI(4,5)P2 and seven models of IP3.

Finally, we used Maestro’s Glide XP docking program (default parameters) (51–54) to position each of the 11 small-molecule models within the candidate binding site associated with each of the two monomers (22 docking runs total).

Docked-pose filtering
Computer docking suggested several candidate PI(4,5)P2- and IP3-binding poses. We used the following criteria to identify the most plausible: discard poses that do not predict reasonable interactions with K446, K592, and R450 (26, 35, 39); discard poses that position any ligand phosphate near the E442 carboxylate; discard poses that are not common to both PI(4,5) P2 and IP3, based on the assumption that the binding mechanisms of both ligands are similar; discard poses that orient the PI(4,5)P2 tail away from the bilayer, based on the assumption that the tail is embedded in the lipid bilayer (55, 56).

Docking visualization
We used PyMOL (Schrödinger, Inc) to visualize all structural models and generate figures. The position of the plasma membrane was retrieved from the OPM Database (57) using 5OYB (23) as a template.

Data availability
The xTMEM16A (X. laevis TransMEMbrane 16A) homology model will be shared upon request (please contact Joel Rosenbaum: jcr80@pitt.edu). All other data are included within the article.

Supporting information—This article contains supporting information.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: cRNA, circular RNA; diC8-PIP3, dioctanoyl phosphatidylinositol 3,4,5-trisphosphate; HSD, phosphatidyl 3,4,5-trisphosphate; TEVC, two-electrode voltage-clamp; TMEM16A, TransMEMbrane 16A; VSP, voltage-sensing phosphatase; XI-VSP, Xenopus laevis voltage-sensing phosphatase.

References


28. Tembo, M., Wozniak, K. L., Bainbridge, R. E., and Carlson, A. E. (2019) Phosphatidylinositol 4,5-bisphosphate (PI(2)P) and Ca(2+)+ are both required to open the Cl− channel TMEM16A. J. Biol. Chem. 294, 12556–12564
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