Two distinct effects of PIP$_2$ underlie auxiliary subunit-dependent modulation of Slo1 BK channels

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Phosphatidylinositol 4,5-bisphosphate (PIP$_2$) plays a critical role in modulating the function of numerous ion channels, including large-conductance Ca$^{2+}$- and voltage-dependent K$^+$ (BK, Slo1) channels. Slo1 BK channel complexes include four pore-forming Slo1 (α) subunits as well as various regulatory auxiliary subunits (β and γ) that are expressed in different tissues. We examined the molecular and biophysical mechanisms underlying the effects of brain-derived PIP$_2$ on human Slo1 BK channel complexes with different subunit compositions that were heterologously expressed in human embryonic kidney cells. PIP$_2$ inhibited macroscopic currents through Slo1 channels without auxiliary subunits and through Slo1 + γ1 complexes. In contrast, PIP$_2$ markedly increased macroscopic currents through Slo1 + β1 and Slo1 + β4 channel complexes and failed to alter macroscopic currents through Slo1 + β2 and Slo1 + β2 β2–19 channel complexes. Results obtained at various membrane potentials and divalent cation concentrations suggest that PIP$_2$ promotes opening of the ion conduction gate in all channel types, regardless of the specific subunit composition. However, in the absence of β subunits positioned near the voltage-sensor domains (VSDs), as in Slo1 and probably Slo1 + γ1, PIP$_2$ augments the negative surface charge on the cytoplasmic side of the membrane, thereby shifting the voltage dependence of VSD-mediated activation in the positive direction. When β1 or β4 subunits occupy the space surrounding the VSDs, only the stimulatory effect of PIP$_2$ is evident. The subunit compositions of native Slo1 BK channels differ in various cell types; thus, PIP$_2$ may exert distinct tissue- and divalent cation–dependent modulatory influences.

INTRODUCTION

Large-conductance Ca$^{2+}$- and voltage-dependent K$^+$ (BK, Slo1) channels are broadly expressed in numerous cell types and regulate many critical physiological processes including neuronal excitability, synaptic transmission, and vascular tone (Salkoff et al., 2006; Hoshi et al., 2013a). To serve these diverse roles, Slo1 BK channels are equipped with multiple mechanisms to increase their functional versatility. For example, Slo1 BK channels can be allosterically activated by both intracellular Ca$^{2+}$ and membrane depolarization (Horrigan and Aldrich, 2002; Hoshi et al., 2013a). Furthermore, native BK channel complexes include four pore-forming Slo1 (α) subunits as well as auxiliary subunits in a tissue-dependent manner (Knaus et al., 1994b; Wallner et al., 1999; Xia et al., 1999; Brenner et al., 2000; Uebele et al., 2000; Yan and Aldrich, 2010, 2012; Yang et al., 2011). Two vertebrate families of Slo1 auxiliary subunits are known: the β-subunit family with four members (β1–β4) (Knaus et al., 1994b; Wallner et al., 1999; Xia et al., 1999; Brenner et al., 2000; Uebele et al., 2000) and the γ-subunit family with multiple members including leucine-rich-repeat-containing (LRRC) protein 26 or γ1 (Yan and Aldrich, 2010, 2012; Yang et al., 2011). Both β and γ subunits are expressed in a tissue-specific manner to fine-tune the properties of the resulting channel complexes. For example, β1-containing BK channels are found abundantly in vascular smooth muscle cells (Knaus et al., 1994b) and promote muscle relaxation (Nelson and Bonev, 2004). β4-containing BK channels are found readily in the nervous system (Behrens et al., 2000; Brenner et al., 2000). Slo1 BK complexes with γ1 are found in testis-related cells and other cell types (Yang et al., 2011; Almassy and Begenisich, 2012; Evanson et al., 2014).

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Abbreviations used in this paper: BK, large-conductance Ca$^{2+}$- and voltage-dependent K$^+$; DHA, docosahexaenoic acid; GR, gating ring; HA model, Horrigan and Aldrich model; LRRC, leucine-rich repeat-containing; PIP$_2$, phosphatidylinositol 4,5-bisphosphate; P_{o}, open probability; VSD, voltage-sensor domain.
Structurally, β subunits contain two transmembrane segments, TM1 and TM2, connected by an extracellular linker, placing the N and C termini on the intracellular side (Knaus et al., 1994a, b). Each β subunit is located most probably between two adjacent voltage-sensor domains (VSDs) (Wu et al., 2009, 2013; Morera et al., 2012; Liu et al., 2015), and up to four β subunits may be found within a tetrameric Slo1 complex (Knaus et al., 1994b; Wang et al., 2002). In contrast with β subunits with two transmembrane segments, γ subunits are processed to contain only one transmembrane segment with its long N-terminal LLRC segment facing the extracellular side (Van and Aldrich, 2010).

To further increase the functional versatility, gating of Slo1 BK channels is modulated by various signaling molecules, such as CO, H⁺, heme, and lipids (Hou et al., 2009). Modulation of Slo1 BK currents by various PIP₂ analogs (C20:4) forms (Balla, 2013). Functionally, PIP₂ plays many cell signaling roles (Balla, 2013). Structurally, PIP₂ possesses a negatively charged inositol head group and two nonpolar fatty acid tails, commonly stearic (C18:0) and arachidonic (C20:4) forms (Balla, 2013). Functionally, PIP₂ directly regulates a variety of ion channels with a diverse range of affinities, typically by binding to positively charged regions of the proteins located near the membrane (Gamper and Shapiro, 2007; Suh and Hille, 2008; Hansen et al., 2011). In many channels, the binding of PIP₂ acts to maintain their functionalities; this is well illustrated in so-called “rundown” phenomena often caused by depletion of PIP₂ in which PIP₂ essentially acts as a functionally necessary cofactor (Balla, 2013). More complex modulatory phenomena by PIP₂ have been also reported. For example, CNG channels in rod photoreceptors are inhibited by PIP₂, whereas CNG channels in olfactory receptors are PIP₂ insensitive (Womack et al., 2000). Furthermore, PIP₂ may exert both excitatory and inhibitory influences in the same channels through multiple interaction sites, as shown in capsaicin-sensitive TRPV1 and hyperpolarization-activated cation channels (Womack et al., 2000; Pian et al., 2006; Flynn and Zagotta, 2011; Hansen et al., 2011).

In Slo1 BK channels, the application of exogenous PIP₂ increases open probability (Pₒ) (Rittenhouse, 2008; Vaithianathan et al., 2008; Tang et al., 2014) and also the number of functional channels available to open after enzymatic depletion of bound PIP₂ (Tang et al., 2014). Exactly which aspect of gating of the Slo1 channel, often described by the multi-tier allosteric model of Horrigan and Aldrich (2002) (HA model), is altered by PIP₂ to increase Pₒ remains unclear. PIP₂ has been suggested to interact with the S6-RCK1 linker region of Slo1 (329RKK-332 Multiple effects of PIP₂ on Slo1 channels using the human Slo1 numbering (Vaithianathan et al., 2008) and also with a segment near the RCK1 Ca²⁺ sensor area (“366KDRDD770 loop”) in the cytoplasmic gating ring (GR) domain (Tang et al., 2014).

We describe here functional impacts of the application of brain-derived PIP₂ on the gating of heterologously expressed human Slo1 BK channels with different auxiliary subunit compositions. We show that the application of PIP₂ can enhance or diminish currents through Slo1 BK channels depending on the subunit composition, and describe two distinct biophysical mechanisms that account for this subunit composition–dependent modulation by PIP₂ based on the probable location of the auxiliary subunits within the Slo1 BK channel complex.

MATERIALS AND METHODS

Channel expression

Human Slo1 (KCNMA1; GenBank accession no. AA658837) and their auxiliary subunits β1 (KCNMB1; RefSeq accession no. NP_0041281), β2 (KCNMB2; RefSeq accession no. NP_852006), β4 (KCNMB4; GenBank accession no. AAF69805), and LLRC26 (RefSeq accession no. NP_001013675) were transiently expressed in human embryonic kidney cells, as described previously (Hoshi et al., 2013c). The cells were maintained in Dulbecco’s modified Eagle’s medium (ATCC), 10% FBS (ATCC), 1% penicillin/streptomycin at 37°C, and 5% CO₂. For those experiments involving coexpression of Slo1 and an auxiliary subunit, the DNA weight ratio in transfection was typically 1:1. Functional coassembly with the auxiliary subunits in each patch was verified by observing the characteristically altered gating properties conferred, such as slowing of the activation and deactivation kinetics (Hoshi et al., 2013b) (see Fig. S5). Electrophysiological characteristics of ionic currents recorded from cells transfected with Slo1 and various auxiliary subunit DNAs together were clearly different from those of Slo1 currents without any auxiliary subunit, suggesting adequate coassembly of Slo1 and the auxiliary subunits.

To express Slo1 in *Xenopus laevis* oocytes, human Slo1 in pC1-neo was linearized with NotI and the RNA was prepared using the T7 RNA polymerase. Surplus oocytes, which were harvested according to an institutionally approved protocol and which would have been otherwise wasted, were obtained from the laboratory of Zhe Lu (University of Pennsylvania, Philadelphia, PA) and injected with the Slo1 RNA.

Electrophysiology and analysis

Currents were recorded using the inside-out patch-clamp method with an AxoPatch 200A or 200B amplifier (Molecular Devices). Borosilicate electrodes coated with dental wax had a typical initial input resistance of 0.9–2 MΩ, and ~60% of the input resistance was electronically compensated. Tight seals were formed as quickly as possible without much negative pressure; only the results from the seals formed within a few seconds were analyzed. The output of the amplifier was filtered through its built-in 10-KHz filter, digitized, and analyzed as described using routines running in IGOR Pro (WaveMetrics) (Horrigan et al., 2005). The external solution typically contained (mM): 140 KCl, 2 MgCl₂, and 10 HEPES, pH 7.4 with NMDG. The internal solution without Ca²⁺ contained (mM): 140 KCl, 11 EGTA, and 10 HEPES, pH 7.2 with NMDG. The solution with different concentrations of Ca²⁺ was as in Horrigan et al. (2005). For each channel type, voltage-pulse durations were adjusted to allow for steady-state measurements. Normalized conductance (G/Gₘₐₓ)–voltage curves were constructed from extrapolated instantaneous tail current
amplitudes. The voltage dependence in each dataset was fitted by a Boltzmann-type equation as described previously (Horrigan et al., 2005) and characterized by two parameters: the half-activation voltage ($V_{0.5}$) and the number of equivalent charges ($Q_{app}$) (Hoshi et al., 2013b,c). PIP$_2$ was applied typically 6–8 min after patch excision after ensuring that the ionic currents were stable in size and kinetics. In the virtual absence of Ca$^{2+}$, confounding associated with rundown was minimal (Zhang et al., 2006). In each patch, changes in voltage dependence of activation by PIP$_2$ were described by changes in half-activation voltage ($\Delta V_{0.5}$) and the fractional change in apparent charge movement ($Q_{app}$ ratio). Estimated equation parameter values are presented as mean ± 95% confidence interval as implemented in IGOR Pro. Kinetics of Slo1 currents were characterized by single exponentials. Estimation of $P_e$ from single-channel openings was performed using all-point amplitude histograms (Horrigan et al., 2005). In each patch, the number of functional channels present was estimated from the peak macroscopic current size at a very positive voltage (e.g., 220 mV) and the unitary current size.

**Statistics**

The numbers of independent measurements are indicated in the figures. Statistical results in the text are presented as mean ± SEM ($n$), where $n$ is the number of independent measurements. The Mann–Whitney or Wilcoxon test with an $\alpha$ level of 0.05, which was corrected for multiple comparisons using the Bonferroni method when appropriate, was used to evaluate statistical significance.

**Online supplemental material**

The concentration dependence and the slow reversibility of brain-derived PIP$_2$ are illustrated in Fig. S1. The results obtained from Slo1 channels expressed in *Xenopus* oocytes are shown in Fig. S2. We show that diC8 PIP$_2$ is less effective than brain-derived PIP$_2$ in Fig. S3. The nonessential role of the Ca$^{2+}$ sensors of the Slo1 channel is presented in Figs. S4 and S6. Adequate coassembly of Slo1 mutants and $\beta_1/\beta_4$ are illustrated in Fig. S5. The online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.201511363/DC1.

**RESULTS**

**Subunit-dependent regulation by brain-derived PIP$_2$**

We systematically compared the effects of the application of brain-derived PIP$_2$ to the cytoplasmic side of Slo1 BK channel complexes with different subunit compositions:

**Reagents**

PIP$_2$ purified from the bovine brain was obtained from Sigma-Aldrich, and diC8 PIP$_2$ was obtained from Echelon Biosciences. Brain PIP$_2$ was dissolved in water (3 mM) by sonication in cold water bath and vigorous vortexing. The stock solution was stored at $-20^\circ$C and diluted to the final concentrations immediately before experiments by vigorous vortexing.

![Figure 1.](https://example.com/figure1.png)

**Figure 1.** Effects of PIP$_2$ on Slo1 complexes with different subunit compositions. (A) Illustrative currents through Slo1, Slo1 + $\beta_1$, Slo1 + $\beta_2$, Slo1 + $\beta_2$ Δ2–19, Slo1 + $\beta_3$, and Slo1 + LRRC26 (γ1). In each panel, currents before (blue) and after (red) the application of 10 µM PIP$_2$ to the cytoplasmic side recorded without Ca$^{2+}$ are shown. Pulses were applied from 0 mV every 3 s except for Slo1 + $\beta_2$, which was stimulated every 10 s. For Slo1 + $\beta_2$, 1-s prepulses to $-100$ mV preceded depolarization pulses. For Slo1 + LRRC26 (γ1), the holding voltage was $-80$ mV. (B) Fractional changes in peak outward currents in Slo1, Slo1 + $\beta_1$, Slo1 + $\beta_2$, Slo1 + $\beta_2$ Δ2–19, Slo1 + $\beta_3$, and Slo1 + LRRC26 (γ1). (C) Normalized conductance (G-V) curves before (blue) and after (red) the application of 10 µM PIP$_2$ in the channels indicated. The smooth curves are Boltzmann fits to the results with: Slo1, $V_{0.5} = 154.5 \pm 3.1$ mV and $Q_{app}$ = 1.33 ± 0.04 (Control), and 170.1 ± 2.7 mV and 1.18 ± 0.05 (PIP$_2$); Slo1 + $\beta_1$, $V_{0.5} = 167.8 \pm 2.0$ mV and $Q_{app}$ = 0.92 ± 0.02 (Control), and 122.3 ± 2.8 mV and 0.95 ± 0.03 (PIP$_2$); Slo1 + $\beta_2$ Δ2–19, $V_{0.5}$ = 163.9 ± 3.0 mV and $Q_{app}$ = 0.98 ± 0.02 (Control), and 158.6 ± 2.4 mV and 0.92 ± 0.02 (PIP$_2$); Slo1 + $\beta_3$, $V_{0.5}$ = 217.2 ± 3.2 mV and $Q_{app}$ = 0.99 ± 0.03 (Control), and 193.2 ± 4.0 mV and 1.00 ± 0.03 (PIP$_2$); and Slo1 + LRRC26 (γ1), $V_{0.5}$ = 20.9 ± 3.5 mV and $Q_{app}$ = 1.38 ± 0.06 (Control), and 42.3 ± 1.8 mV and 1.09 ± 0.04 (PIP$_2$); $n$ = 9–18. Error bars represent mean ± SEM.
Multiple effects of PIP2 on Slo1 channels

Slo1 without any auxiliary subunit (hereafter referred to as Slo1), Slo1 + β1, Slo1 + β2, Slo1 + β4, and Slo1 + LRRC26 (γ1) (Knaus et al., 1994b; Wallner et al., 1999; Xia et al., 1999; Brenner et al., 2000; Uebele et al., 2000; Yan and Aldrich, 2010, 2012; Yang et al., 2011). We also examined the effect of PIP2 on Slo1 + β2 with a deletion in the β2 N terminus (Δ2–19) to remove inactivation (Wallner et al., 1999; Uebele et al., 2000; Xia et al., 2003). Representative macroscopic ionic currents recorded from the aforementioned channel complexes before and after application of 10 μM PIP2 in the virtual absence of Ca²⁺ to the intracellular side are shown in Fig. 1 A. This concentration was a functionally saturating “bulk” concentration of PIP2; 30 μM caused no further change (Fig. S1). PIP2 clearly decreased macroscopic currents through Slo1 and Slo1 + γ1 but increased macroscopic currents through Slo1 + β1 and Slo1 + β4 (Fig. 1, A and B). PIP2 had no appreciable effect on Slo1 + β2 Δ2–19 (Fig. 1, A and B). The differential impacts of PIP2 on these channel types were also evident in their voltage dependence of normalized conductance (G/Gmax) curves (G-V curves) before and after the application of PIP2 (Fig. 1 C). In Slo1, PIP2 shifted the half-activation voltage (V0.5) of G-V to the positive direction by 15.6 ± 2.6 mV (9). In contrast, in Slo1 + β1 and Slo1 + β4, PIP2 moved the G-V curves to the negative direction (ΔV0.5 = −45.5 ± 2.1 mV [18] and −33.9 ± 2.8 mV [16], respectively). Slo1 + β2 Δ2–19 showed no change in V0.5 (ΔV0.5 = −5.3 ± 2.1 mV [18]), whereas Slo1 + γ1 showed a positive shift of V0.5 (ΔV0.5 = 21.4 ± 3.3 mV [9]), similar to that observed in Slo1. These effects of PIP2 were very poorly reversible by wash (Fig. S1, A and C).

PIP2 also altered the ionic current kinetics in a subunit composition–dependent manner (Fig. 2). The current enhancement by PIP2 observed in Slo1 + β1 and Slo1 + β4 was accompanied by deceleration of the kinetics at the voltages where G/Gmax is less than ~0.5 and acceleration of the kinetics at more positive voltages. In Slo1 and Slo1 + γ1, in which the currents were inhibited, PIP2 preferentially slowed the kinetics at the voltages where G/Gmax is greater than ~0.5.

Next, we will focus on the following three subunit-dependent phenomena caused by brain-derived PIP2: (1) macroscopic current inhibition in Slo1 but current enhancement in Slo1 + β1 and Slo1 + β4 (Fig. 1, A and B), (2) macroscopic current enhancement in Slo1 + β1 and Slo1 + β4 but no marked effect in Slo1 + β2 Δ2–19 (Fig. 1, A and B), and (3) the molecular loci responsible for the effects of PIP2 on Slo1 and Slo1 + β1.

Slo1 versus Slo1 + β1

In Slo1, PIP2 shifts its V0.5 to the positive direction and decreases macroscopic currents at voltages >120 mV (Fig. 1 B). In contrast, in Slo1 + β1, PIP2 shifts its V0.5 to the negative direction and enhances macroscopic currents (Fig. 1 B). A similar inhibitory effect of PIP2 was...
observed using Slo1 channels heterologously expressed in *Xenopus* oocytes (Fig. S2). The application of diC8 PIP₂, a more water-soluble PIP₂ analogue with short eight-carbon tails, produced no effect on Slo1 but increased currents through Slo1 + β1, albeit to a lesser extent (Fig. S3), suggesting that the long tail groups of brain-derived PIP₂ contribute to its action on Slo1 and Slo1 + β1 channels, as seen in an earlier study (Vaithianathan et al., 2008).

Gating of the Slo1 channel is allosteric, encompassing three gating tiers, the ion conduction gate, VSDs, and divalent cation sensors, as summarized in the HA model (Horrigan and Aldrich, 2002). The three allosteric tiers can be studied in relative isolation by manipulating membrane potential and intracellular Ca²⁺ concentration ([Ca²⁺]i) (Horrigan and Aldrich, 2002). In the absence of Ca²⁺ at very negative voltages where the VSDs are mostly at rest, PIP₂ increased P₀ in both Slo1 (Fig. 3, A and C) and Slo1 + β1 (Fig. 3, B and C), and the fractional increases were indistinguishable between the two channel types (P = 0.955; Fig. 3 D), despite the contrasting effects at more positive voltages where VSD activation and G/G_max are appreciable. P₀ at negative voltages without Ca²⁺ primarily reflects the weakly voltage-dependent equilibrium of the ion conduction gate (L₀ in the HA model). According to the HA model, an increase in the value of L₀ as suggested by our single-channel P₀ measurements predicts a negative shift in V₀.₅. For Slo1 + β1, this prediction is born out; however, for Slo1, whose macroscopic currents are inhibited by PIP₂, the prediction obviously does not hold. We postulated that the inhibitory effect of PIP₂ on Slo1 at positive voltages where VSD activation and G/G_max are noticeable may represent a separate phenomenon superimposed on the common stimulatory effect readily observed at negative voltages without Ca²⁺ in both Slo1 and Slo1 + β1.

Membrane phospholipids with charged head groups are capable of altering functions of voltage-gated ion channels by directly interacting with the VSD and/or by influencing the membrane surface charge (Ramu et al., 2006; Xu et al., 2008; Hite et al., 2014). We hypothesized that the application of PIP₂ with the net charge of about −4 (Wang et al., 2014) to the intracellular side augments the negative surface charge on the intracellular membrane surface, and that this enhanced negative surface charge may underlie the inhibitory influence of PIP₂ on the macroscopic currents through Slo1. The surface-charge effect on the VSDs at the intracellular side of the channel would then counteract the stimulatory effect of PIP₂ mediated by a distinct molecular locus (see below). According to this idea, screening of the additional negative charges provided by PIP₂, for example by Mg²⁺, should only leave the stimulatory effect operative. The results of an illustrative experiment manipulating the intracellular Mg²⁺ concentration are depicted in Fig. 4 (A and B). Without added divalent cations, 10 µM PIP₂ caused an inhibitory positive shift of V₀.₅ (ΔV₀.₅ = 19.8 ± 2.8 mV [9]) in the mutant Slo1 channel whose intracellular divalent cation sensors are disrupted (D362A:D367A:E399A:894–895; Fig. 4 A, red) (Xia et al., 2002; Zhang et al., 2010). But subsequent application of 10 mM Mg²⁺ reversed the shift direction caused by PIP₂ (ΔV₀.₅ = −27.6 ± 2.8 mV [8] compared with the original condition without PIP₂) and increased currents, presumably by screening the negative surface charges provided by PIP₂ (Fig. 4 A, gray). A smaller physiological concentration of Mg²⁺ (2 mM) was also effective in reversing the “polarity” of the effect.

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![Figure 3](http://example.com/figure3.png)

**Figure 3.** PIP₂ increases P₀ at negative voltages without Ca²⁺. (A) Representative single-channel openings at −120 mV of Slo1 without Ca²⁺ before and after the application of 10 µM PIP₂. In each condition, 25 data traces are shown superimposed. This patch contained ~350 channels. (B) Representative single-channel openings at −120 mV of Slo1 + β1 without Ca²⁺ before and after the application of 10 µM PIP₂. 60 data traces are shown superimposed, and the patch contained ~250 channels. (C) Comparison of P₀ changes in Slo1 and Slo1 + β1 by 10 µM PIP₂. (D) Fractional changes in P₀ by 10 µM PIP₂; n = 7 and 8 for Slo1 and Slo1 + β1, respectively. All results were obtained without Ca²⁺. Error bars represent mean ± SEM.
Multiple effects of PIP2 on Slo1 channels

In addition to Mg$^{2+}$, Ca$^{2+}$ was similarly effective (Fig. 4 D). With 100 µM Ca$^{2+}$, a saturating concentration for the high affinity divalent cation sensors of the channel (Horrigan and Aldrich, 2002), PIP2 enhanced currents through Slo1 D362A:D367A:E399A:894–895 (Fig. 4 D; $\Delta V_{0.5} = -25.6 \pm 3.7$ mV [5]) and those through wild-type Slo1 (Fig. 4 E; $\Delta V_{0.5} = -22.2 \pm 3.7$ mV [7]) by shifting $V_{0.5}$ to the negative direction. The measurements manipulating the concentration of Ca$^{2+}$ showed that the crossover from the stimulatory effect ($\Delta V_{0.5} < 0$) to the inhibitory effect ($\Delta V_{0.5} > 0$) of PIP2 occurs around a few micromolars of [Ca$^{2+}$] (Fig. 4, F and G). The effectiveness of PIP2 with 100 µM Ca$^{2+}$ also indicates that the stimulatory action of PIP2 does not require modulation of the high affinity Ca$^{2+}$ sensor activation. When β1 was coexpressed, a robust stimulatory effect of PIP2 clearly persisted with 10 mM Mg$^{2+}$ (Slo1 D362A:D367A:E399A:Δ894–895 + β1; Fig. 4, H and I) and also with 100 µM Ca$^{2+}$ (wild-type Slo1 + β1; Fig. 4 J). A much greater concentration of Ca$^{2+}$ (2 mM) was required to antagonize the stimulatory effect of PIP2 in Slo1 D362A:D367A:E399A:Δ894–895 + β1 (Fig. S4), suggesting that the stimulatory effect of PIP2 may be mediated by a tighter interaction, potentially involving a PIP2-binding pocket (Suh and Hille, 2008).

If excess negative charges on the intracellular membrane surface underlie the positive shift of $V_{0.5}$ by PIP2 in Slo1, excess negative charges on the extracellular surface should produce a negative shift of $V_{0.5}$, thereby enhancing currents through Slo1. Consistent with this prediction, the application of PIP2 to the extracellular side indeed caused a clear negative shift of $V_{0.5}$ ($-14.5 \pm 1.2$ mV [6]; red) the application of 10 µM PIP2 in the presence of 2 mM Mg$^{2+}$. (D) Representative currents (left) and G-V curves from five patches (right) containing wild-type Slo1 before (blue) and after (red) the application of 10 µM PIP2 in the presence of 100 µM Ca$^{2+}$. (E) Representative currents (left) and G-V curves from seven patches (right) containing wild-type Slo1 before (blue) and after (red) the application of 10 µM PIP2 in the presence of 100 µM Ca$^{2+}$. (F and G) Ca$^{2+}$ dependence of $V_{0.5}$ before (blue) and after (red) the application of 10 µM PIP2 (F) and that of $\Delta V_{0.5}$ (G) by 10 µM PIP2. (H) Representative currents from Slo1 D362A:D367A:E399A:Δ894–895 + β1 before (blue) and after (red) the application of 10 µM PIP2 in the presence of 10 mM Mg$^{2+}$. (I) Changes in $V_{0.5}$ of Slo1 D362A:D367A:E399A:Δ894–895 + β1 by PIP2 and Mg$^{2+}$; $n = 8$. (J) Representative currents (left) and G-V curves from six patches (right) containing wild-type Slo1 recorded in the outside-out configuration before (blue) and after (red) the application of 10 µM PIP2 with 100 µM Ca$^{2+}$ inside. (K) Representative currents (left) and G-V curves from six patches (right) containing wild-type Slo1 recorded in the outside-out configuration before (blue) and after (red) the application of 10 µM PIP2 to the extracellular side without any added Mg$^{2+}$ or Ca$^{2+}$. The $V_{0.5}$ values before and after the application of PIP2 were 151.7 ± 1.7 mV and 137.2 ± 1.3 mV (P = 0.0021; $n = 6$). Error bars represent mean ± SEM.

Figure 4. Manipulations of divalent cation concentrations alter the direction of the PIP2 effect in Slo1. (A) G-V curves before (blue) and after PIP2 addition (red), and the subsequent addition of 10 mM Mg$^{2+}$ (black), from a representative patch expressing divalent cation-insensitive Slo1 D362A:D367A:E399A:Δ894–895 channels. (B) Changes in $V_{0.5}$ of Slo1 D362A:D367A:E399A:Δ894–895 by PIP2 and Mg$^{2+}$. (C) Representative currents (left) and G-V curves from five patches (right) of wild-type Slo1 before (blue) and after (red) the application of 10 µM PIP2 in the presence of 100 µM Mg$^{2+}$; $n = 8$. (F and G) Ca$^{2+}$ dependence of $V_{0.5}$ before (blue) and after (red) the application of 10 µM PIP2 (F) and that of $\Delta V_{0.5}$ (G) by 10 µM PIP2. (H) Representative currents from Slo1 D362A:D367A:E399A:Δ894–895 + β1 before (blue) and after (red) the application of 10 µM PIP2 in the presence of 10 mM Mg$^{2+}$. (I) Changes in $V_{0.5}$ of Slo1 D362A:D367A:E399A:Δ894–895 + β1 by PIP2 and Mg$^{2+}$; $n = 8$. (J) Representative currents (left) and G-V curves from six patches (right) containing wild-type Slo1 recorded in the outside-out configuration before (blue) and after (red) the application of 10 µM PIP2 with 100 µM Ca$^{2+}$ inside. (K) Representative currents (left) and G-V curves from six patches (right) containing wild-type Slo1 recorded in the outside-out configuration before (blue) and after (red) the application of 10 µM PIP2 to the extracellular side without any added Mg$^{2+}$ or Ca$^{2+}$. The $V_{0.5}$ values before and after the application of PIP2 were 151.7 ± 1.7 mV and 137.2 ± 1.3 mV (P = 0.0021; $n = 6$). Error bars represent mean ± SEM.
contains the same number of residues as in β1 (Fig. 5 A). In Slo1 + β2 Δ2–32, PIP2 caused a noticeable negative shift in GV (ΔV0.5 = −28.8 ± 2.3 mV [17]), whereas PIP2 had essentially no effect in Slo1 + β2 Δ2–19 (P < 10−7; but smaller than that in Slo1 + β1, P < 10−6; Fig. 5 B). This observation suggests that the stimulatory action of PIP2 observed in Slo1 + β2 Δ2–32 is impaired by the differences in the segment between β2 Δ2–19 and β2 Δ2–32 (residues 20–32; Fig. 5 A). This segment possesses

Figure 5. Critical role of the β N terminus in determining ΔV0.5 by PIP2. (A) Sequence alignment of β1, β2 Δ2–19, and β2 Δ2–32 N termini. (B) Changes in G-V parameters by PIP2 in Slo1 complexes with different β subunits. (C) Sequence alignment of β1, β2–32, and β4 N termini. (D) Changes in G-V parameters by PIP2 in Slo1 + β1 complexes with the β1-to-β2 point mutations indicated. (E) Changes in G-V parameters by PIP2 in Slo1 + β1 complexes with the β1-to-β2 point mutations at position 11. (F) Changes in G-V parameters by PIP2 in Slo1 + β2 with the β2-to-β1 point mutations indicated. (G) Changes in G-V parameters by PIP2 in Slo1 + β2 with the β4-to-β2 (bottom) point mutations indicated. In D–F, the gray shaded areas represent the mean ± SEM of ΔV0.5 by PIP2 in Slo1 + β1 (left) and Slo1 + β2 Δ2–32 (right). In G, the gray shaded area shows the mean ± SEM of ΔV0.5 by PIP2 in Slo1 + β2 Δ2–32. All results were obtained without Ca2+. Error bars represent mean ± SEM.
multiple positively charged residues (K24, R26, and H28 using full β2 numbering) and negatively charged residues (D27, D29, and D32), which could potentially interact with the negatively charged head group of PIP2. Neutralization of the positively charged residues in β2 Δ2–19 (β2 Δ2–19 K24N:R26N:H28N) did not confer a greater shift of V0.5 by PIP2 to Slo1 + β2 Δ2–19 (Fig. 5 B). However, neutralization of the negatively charged residues (β2 Δ2–19 D27Q:D29Q:D32Q) significantly increased ΔV0.5 by PIP2 to −18.6 ± 1.0 mV (8) (P = 0.00017; Fig. 5 B), indicating that there may be an electrostatic repulsion between these negatively charged residues and the PIP2 head group. The PIP2-induced ΔV0.5 in Slo1 + β2 Δ2–19 K24N:R26N:D27Q:H28N:D29Q:D32Q, in which both the positively charged residues and negatively charged residues are neutralized, was indistinguishable

![Diagram](Figure 6. Roles of β2 KKK and the GR domain in modulation of P0 in Slo1 + β1 by PIP2. (A) Schematic structural organization of a Slo1 subunit without the GR domain (Slo1ΔGR-Kv-minT). In Slo1ΔGR-Kv-minT, the polypeptide is truncated immediately C terminal to the sequence β2 KKK. The distal red segment represents the amino acids added by Budelli et al. (2013) (GKESLG-GTDV). (B) Representative currents from Slo1ΔGR-Kv-minT + β1 before (blue) and after (red) the application of 10 µM PIP2. (C) Fractional changes in peak outward currents at different voltages by 10 µM PIP2 (red). The gray shaded area shows the mean ± SEM results from wild-type Slo1 + β1 for comparison. (D, F, and H) Illustrative currents through Slo1 R329A:K330A:K331A (D), Slo1 R329A:K330A:K331A + β1 (F), and Slo1 R329A:K330A:K331A + β4 (H) before (blue) and after (red) the application of 10 µM PIP2. (E, G, and I) G-V curves of Slo1 R329A:K330A:K331A (“KKK mutant”), Slo1 R329A:K330A:K331A (“KKK mutant”) + β1, and Slo1 R329A:K330A:K331A (“KKK mutant”) + β4 before (blue circles) and after (red circles) the application of 10 µM PIP2. For comparison, G-V curves from the respective wild-type Slo1 (E), Slo1 + β1 (G), and Slo1 + β4 (I) before (blue triangles) and after (red triangles) the application of 10 µM PIP2 are also shown; n = 6–8. All results were obtained without Ca2+. Error bars represent mean ± SEM.)
from that in Slo1 + β2 Δ2–19 D27Q:D29Q:D32Q (P = 0.53; Fig. 5 B).

The difference in Δ$V_{0.5}$ between Slo1 + β2 Δ2–19 and Slo1 + β2 Δ2–32 is therefore accounted for largely by the negatively charged residues D27, D29, and D32; however, the Δ$V_{0.5}$ values in Slo1 + β2 Δ2–32 and Slo1 + β1 still differ significantly (Fig. 5 B). Similar contrasting behavior between Slo1 + β2 Δ2–19/Δ2–32 and Slo1 + β1 was observed with the stimulatory effect of the omega-3 fatty acid docosahexaenoic acid (DHA), and the N-terminal residues in β1 and β2 Δ2–32 (Fig. 5 C) were found to be critical (Hoshi et al., 2013b). We thus examined if the N termini of β1 and β2 Δ2–32 also contributed to the difference in PIP$_2$-induced Δ$V_{0.5}$ in Slo1 + β1 and Slo1 + β2 Δ2–32 by introducing β1-to-β2 single-residue mutations to β1 (Figs. 5, C and D, and S5). The β1-to-β2 mutations V2K, V1T, M7T, and Q9L in the β1 background failed to alter Δ$V_{0.5}$ by PIP$_2$. The β1-to-β2 mutations R11A and T14D in β1 diminished the Δ$V_{0.5}$ of the resulting Slo1 + β1 complexes by PIP$_2$ from −45 mV to −27.9 ± 3.6 mV (8) and −34.5 ± 2.5 mV (11), respectively, which were indistinguishable from that observed in Slo1 + β1 Δ2–32 (P = 0.80 and 0.09, respectively). A higher bulk concentration of PIP$_2$ (30 µM) did not produce a greater change (P = 0.80 and 0.09, respectively). A higher bulk concentration of PIP$_2$ (30 µM) did not produce a greater change (P = 0.80 and 0.09, respectively). A higher bulk concentration of PIP$_2$ (30 µM) did not produce a greater change (P = 0.80 and 0.09, respectively).

The critical nature of Arg at position 11 in β1 is further suggested by the converse β2-to-β1 mutation A42R in the β2 background in which Ala at position 42 of β2, equivalent to position 11 in β1 (Fig. 5 F), is substituted with Arg as found in β1. This point mutation conferred a greater Δ$V_{0.5}$ by PIP$_2$ to Slo1 + β2 Δ2–32 (−41.5 ± 1.4 mV [6]), indistinguishable from that found in Slo1 + β1 (P = 0.38). The mutation β2 D45T, the β2-to-β1 mutation at position 45 in β2, equivalent to position 14 in β1 (Fig. 5 C), did not produce a functional β2 subunit. The double mutant β2 A42R:D45T was functional and the Δ$V_{0.5}$ by PIP$_2$ in Slo1 + β2 A42R:D45T did not differ from that in Slo1 + β1 A42R (Fig. 5 F; P = 0.35). Mutation of charged Glu at position 12 in β2, equivalent to position 11 in β1, to Arg (E12R) as in β1 (Fig. 5 C), enhanced the Δ$V_{0.5}$ in the Slo1 + β4 from −34 mV to −60.6 ± 4.7 mV (5), which is even greater than that in Slo1 + β1 (Fig. 5 G; P = 0.007). In contrast, the β4-to-β2 mutation β4 E12A did not alter Δ$V_{0.5}$ by PIP$_2$ in Slo1 + β4 (Fig. 5 G; P = 0.78). Collectively, the presence of Arg at positions equivalent to position 11 in β1 clearly plays a pivotal role in determining Δ$V_{0.5}$ by PIP$_2$ in Slo1 + β complexes.

**Essential molecular loci**

Two areas of the Slo1 channel have been implicated in the stimulatory effects of PIP$_2$ in earlier studies: 329RKK$^{331}$ immediately C terminal to S6 but N terminal to the RCK1 segment (Vaithianathan et al., 2008), and the 360KDRDD$^{360}$ loop in the RCK1 Ca$^{2+}$-sensor area in the GR domain (Tang et al., 2014). The contribution of the GR domain including the RCK1 Ca$^{2+}$-sensor area and its vicinity to the PIP$_2$-mediated regulation was assessed using the truncated Slo1 channel without the GR domain but with the 329RKK$^{331}$ sequence intact (Slo1ΔGR-Kv-minT; Budelli et al., 2013; Fig. 6 A). The voltage dependence of Slo1ΔGR-Kv-minT + β1 was shifted markedly to the positive direction, and an accurate determination of its G-V was not practical. However, PIP$_2$ unmistakably increased currents through Slo1ΔGR-Kv-minT + β1 (Fig. 6, B and C); the GR domain is not required for the stimulatory action of PIP$_2$ on the Slo1 + β1 complex. Furthermore, PIP$_2$ also remained effective in activating Slo1 D362A:D367A:E399A:Δ349–895 + β1, in which both the high affinity and low affinity divalent cation sensors are impaired (Xia et al., 2002; Zhang et al., 2010) (Δ$V_{0.5}$ = −53.3 ± 5.8 mV [14]; P = 0.16 compared with WT Slo1 + β1; Fig. S6).

The sequence 329RKK$^{331}$ immediately C terminal to S6 has also been implicated in the action of PIP$_2$ (Vaithianathan et al., 2008). The mutation Slo1 R329A:K330A:K331A was reported to diminish the stimulatory effect of PIP$_2$ when measured with an intermediate [Ca$^{2+}$]$_i$ (0.3 µM) (Vaithianathan et al., 2008). Our measurements without Ca$^{2+}$ with Slo1 R329A:K330A:K331A + β1 revealed that the mutation drastically shifted the voltage dependence to the negative direction (Fig. 6, F and G). The application of PIP$_2$ failed to enhance currents through Slo1 R329A:K330A:K331A + β1 and caused a positive shift of $V_{0.5}$ (Δ$V_{0.5}$ = 22.9 ± 5.1 mV [6]; P < 10$^{-4}$ compared with Slo1 + β1; Fig. 6, F and G). In Slo1 R329A:K330A:K331A (Fig. 6, D and E) and Slo1 R329A:K330A:K331A + β4 (Fig. 6, H and I), PIP$_2$ failed to alter $V_{0.5}$ (P = 0.64 and 0.31, respectively).

**DISCUSSION**

Slo1 BK channels are widely expressed in different tissues and play important roles in numerous physiological phenomena (Salkoff et al., 2006; Hoshi et al., 2013a). Two of the important factors contributing to the functional versatility of Slo1 BK channels are tissue-dependent inclusion of auxiliary subunits and modulation of their gating by different signaling molecules (Salkoff et al., 2006; Hou et al., 2009; Hoshi et al., 2013a). Our study here demonstrates that brain-derived PIP$_2$, most probably with stearic and arachidonic tails (Balla, 2013), regulates Slo1 BK channels in a subunit composition–dependent manner through two distinct biophysical mechanisms. At the voltages where VSD activation and G/G$_{\text{max}}$ are appreciable (e.g., >0.05), PIP$_2$ inhibits currents through Slo1 and Slo1 + γ1 but markedly increases currents
through Slo1 + β1 and to a lesser extent those through Slo1 + β4.

The inhibitory effect of PIP2 on macroscopic currents through Slo1 in patches taken from human embryonic kidney cells and Xenopus oocytes is in contrast with the results obtained by Vaithianathan et al. (2008), who showed a noticeable increase in P0 of Slo1 (without any auxiliary subunit) heterologously expressed in Xenopus oocytes and also of native Slo1 in skeletal muscle cells. Using Slo1 without auxiliary subunits expressed in Xenopus oocytes, Tang et al. (2014) also observed a current-enhancing effect of PIP2 after pretreatment with the phosphoinositide 3-kinase inhibitor wortmannin (25 µM for ≥2 h). The exact reasons for the divergent observations remain unclear. It is possible that PIP2 exerts multiple actions, some of which are observed preferentially under different experimental conditions. Perhaps seemingly subtle differences in cell culturing/preparation methods, potentially affecting the membrane lipid composition (Epand, 2008), may also underlie the contrasting observations.

Our electrophysiological measurements manipulating membrane potential and [Ca2+]i, to isolate specific aspects of gating of the Slo1 BK channel complex suggest that the application of exogenous brain-derived PIP2 induces two separate effects with different divalent cation sensitivities depending on the subunit composition. The effect of PIP2 observed in both Slo1 and Slo1 + β1, and most probably in all others, is to bias the equilibrium of the ion conduction gate, L0, in the HA model (Horrigan and Aldrich, 2002), toward the open state. Such a change shifts V0.5 to the negative direction as observed in Slo1 + β1, Slo1 + β2 Δ2-32, and Slo1 + β4. This increase in L0 is probably mediated by the binding of PIP2 to the sequence 329RKK331 immediately C terminal to S6 because the Slo1 mutation R329A:K330A:K331A (Vaithianathan et al., 2008) appears to disrupt the stimulatory effect of PIP2 on Slo1 + β1. It should be noted, however, that the mutation itself without any exogenous PIP2 strikingly shifts the voltage dependence of activation to the negative direction, and some interpretational uncertainty exists. The interaction between the sequence 329RKK331 and the negative charges of brain-derived PIP2 is stabilized by its long 18- and 20-carbon tail groups, most probably in the membrane, causing a near irreversible action of PIP2. In contrast, diC8 with short tails produces a smaller and more reversible effect. Quantum and molecular dynamics simulations suggest that Ca2+ interacts with the negative charges of PIP2 more closely than Mg2+ (Slochover et al., 2013). Consistent with the simulation results, we find that 2 mM Ca2+ disrupts the stimulatory effect of PIP2 on P0 of Slo1 + β1, but the increase persists with 10 mM Mg2+. Besides the sequence 329RKK331, the RCK1 Ca2+ sensor in the GR domain has been implicated in the stimulatory action of PIP2 (Tang et al., 2014). However, an essential role of the GR domain may be excluded, at least in our experimental conditions, because the truncated Slo1 channel without the GR domain can be robustly stimulated by PIP2 when coexpressed with β1.

Multiple studies suggest that the S6-RCK1 linker segment containing the sequence 329RKK331 is a critical structural determinant of Slo1 gating. This segment has been suggested to function as a passive mechanical spring affecting the overall voltage dependence of channel activation (Niu et al., 2004). The mutation of 329RKK331, which alters the response to PIP2 (Vaithianathan et al., 2008) (Fig. 6), produces a very large shift in voltage dependence of activation (Fig. 6). Furthermore, the segment also regulates the sensitivity of the channel to so-called BK openers such as the dehydroabietic acid derivative Cym04 and NS1619, both of which increase L0 (Gessner et al., 2012) as found for PIP2. The interaction of PIP2 with the sequence 329RKK331 may facilitate the rotational movement of the S6 side chains that is speculated to accompany the opening of the ion conduction gate (Chen et al., 2014). How this may occur remains an open question.

In Slo1, but not in those with β subunits, PIP2 inhibits ionic currents at the voltages where VSD activation is appreciable by providing additional negative surface charges on the intracellular surface of the membrane, as suggested by the results of our experiments designed to screen the negative surface charges provided by PIP2 with Mg2+ (Fig. 4). 2 mM Mg2+ or 100 µM Ca2+ is sufficient to antagonize this surface charge effect. Assuming that the transmembrane segments of Slo1 are organized like those in Kv1.2/2.1 (Long et al., 2007), one VSD in Slo1 is separated from an adjacent VSD by membrane lipids. We postulate that PIP2 molecules may position in this cleft area between two neighboring VSDs and provide additional negative charges that could be sensed by the VSDs, thus altering the voltage-sensor equilibrium of the channel. Extracellular disulfide cross-linking studies suggest that the two transmembrane segments of β subunits occupy this same area between the adjacent VSDs (Wu et al., 2009, 2013; Morera et al., 2012; Liu et al., 2015). We propose that this positioning of β subunits prevents the negative charges of PIP2 molecules from closely approaching the VSDs while allowing for the interaction of PIP2 with the sequence 329RKK331 to increase L0. The three-dimensional structural location of the sequence 329RKK331 within Slo1 is unknown; however, we speculate that these residues may be situated near the radial periphery of the channel such that PIP2 has a ready access. Unlike β subunits with two membrane-spanning segments, γ1 with one membrane-spanning segment (Yan and Aldrich, 2010) may allow PIP2 to approach the VSDs; currents through Slo1 + γ1 at positive voltages are inhibited by PIP2 applied to the intracellular side as in Slo1. One unexpected set of findings concerns Slo1 R329A:K330A:K331A, in which the sequence...
is neutralized. Without any β or γ subunit, PIP2 was expected to cause a positive shift in voltage dependence of activation in Slo1 R329A:K330A:K331A, but the observed shift is negligible. Additionally, in Slo1 R329A:K330A:K331A + β1, in which juxtaposition of PIP2 and the VSDs was expected to be impaired by β1, PIP2 induces a positive shift. In contrast, the results from Slo1 R329A:K330A:K331A + β4 are in line with our expectation. Some of the results using Slo1 R329A:K330A:K331A are thus difficult to interpret in part because of the severe basal phenotype of this mutant.

In Slo1 + β complexes, the β N terminus plays a critical role in determining the extent of current enhancement by PIP2. Our mutagenesis results show that three negatively charged Asp residues in the β2 N terminus at positions 27, 29, and 32 impair the electrophysiological response of the channel complex to PIP2. We propose that an electrostatic repulsion between these β2 Asp residues and the PIP2 head group may exist, interfering with the interaction of the PIP2 head group with the probable effector sequence 329RKK331 in Slo1. In addition to the Asp residues in the β2 N terminus, Arg at position 11 of β1 is also critical. Even a charge-conserved substitution with Lys at this position markedly diminishes the response of the channel complex to PIP2. At the extracellular side, β TM1 is probably 2 to 3 nm away from Slo1 S6 (Wu et al., 2009, 2013; Liu et al., 2010, 2015), and the β N terminus with ~15 residues could position itself near the cytoplasmic end of S6 or the S6-RCK1 segment. Asp at position 45 in β2, equivalent to position 14 in β1, has been suggested to interact with the residues near the RCK1 Ca2+ sensor (Hou et al., 2013). The importance of Arg at position 11 was also suggested for the stimulatory effect of DHA on Slo1 + β1 mediated by an increase in L0 (Hoshi et al., 2013b). Although both DHA and PIP2 increase L0 in Slo1 + β1, the mutation R11K in β1 preserves the wild-type-like response to DHA (Hoshi et al., 2013b) but not to PIP2. Thus, the structural interactions involving Arg at position 11 in β1 required for the effects of DHA and PIP2 appear to be different.

PIP2 regulates numerous proteins including ion channels with different affinities (Suh and Hille, 2008). Some channels have very high affinities for PIP2, which essentially acts as a co-factor necessary for proper functionality (Suh and Hille, 2008). For others, PIP2 acts as a dynamic reversible modulator of their functions (Suh and Hille, 2008). In Slo1 BK channels, PIP2 has multiple sites of action, targeting different functional aspects: the number of channels available to open (Tang et al., 2014), Ca2+-dependent activation (Tang et al., 2014), voltage-dependent activation (this study), and intrinsic gating of the ion conduction gate (this study). The finding that the PIP2-mediated modulation depends strongly on the auxiliary subunit composition suggests that PIP2 regulates Slo1 BK channels in a tissue-dependent manner, further increasing the functional versatility of the channels.

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Figure S1. Concentration dependence and slow reversibility of the PIP₂ action. (A) Peak outward current size at 150 mV as a function of time before the application of PIP₂ (blue), in the presence of 10 and 30 µM PIP₂ (red), and after washout of PIP₂ (black) in Slo1 channels. (B) Current-enhancing effect of PIP₂ in Slo1+ β1 is functionally saturated at 10 µM. Representative currents at 120 mV before and after the application of 10 and 30 µM PIP₂ (top) and peak outward current size at 120 mV (bottom). (C) Illustrative currents (top) and peak outward current size (bottom) recorded from Slo1+ β1 before the application of PIP₂ (blue), in the presence of 10 µM PIP₂ (red), and after washout of PIP₂ (black). Currents were recorded without Ca²⁺.

Figure S2. Inhibitory effect of PIP₂ on Slo1 channels expressed in Xenopus oocytes. (A) Representative currents through Slo1 from a patch taken from a Xenopus oocyte before (blue) and after (red) the application of 10 µM PIP₂. (B) Representative peak I-V curves from Slo1 expressed in a Xenopus oocyte before (blue) and after (red) the application of 10 µM PIP₂. (C) G-V curves from Slo1 expressed in Xenopus oocytes before (blue) and after (red) the application of 10 µM PIP₂. The V₅₀ and Qₑₑₑₑ values for the control group are 177.6 ± 5.5 mV and 1.45 ± 0.15, and for the PIP₂ group, they are 192.6 ± 4.5 mV and 1.00 ± 0.06, respectively; n = 4.
Figure S3. diC8 PIP2 is less effective than brain-derived PIP2 on both Slo1 and Slo1 + β1. (A) Representative currents through Slo1 before (blue) and after (red) the application of 10 µM diC8. (B) G-V curves of Slo1 before (blue) and after (red) the application of 10 µM diC8 PIP2. The smooth curves are Boltzmann fits to the results. The V_{0.5} and Q_{app} values are 168.4 ± 1.8 mV and 1.17 ± 0.06 for control and 173.3 ± 3.8 mV and 1.19 ± 0.06 for the application of diC8 PIP2 (n = 6). (C) Represent currents through Slo1 + β1 before (blue) and after (red) the application of 10 µM diC8. (D) Peak outward currents through Slo1 + β1 elicited by pulses to 120 mV as a function of time. The red bar indicates the diC8 PIP2 application period. (E) Illustrative Slo1 + β1 G-V curves before (blue), during application of 10 µM diC8 PIP2 (red), and after washout (black). The V_{0.5} and Q_{app} values are 188 mV and 0.84 for the control group, 158 mV and 0.95 for the PIP2 group, and 174 mV and 0.84 for the washout group, respectively. (F) Comparison of ΔV_{0.5} in Slo1 + β1 by 10 µM of brain-derived PIP2 (blue) and 10 µM diC8 PIP2 (red). All results shown were obtained without Ca^{2+}. Error bars represent mean ± SEM.

Figure S4. A high concentration of Ca^{2+} antagonizes the stimulatory effect of PIP2 on Slo1 D362A:D367A:E399A:Δ894–895 + β1. (A) Peak outward currents through Slo1 D362A:D367A:E399A:Δ894–895 + β1 elicited by pulses to 160 mV. The application of 2 mM Ca^{2+} to the intracellular side antagonizes the stimulatory effect of 10 µM PIP2, and the antagonistic effect of Ca^{2+} is relieved by the Ca^{2+} chelator EGTA (11 mM). (B) Illustrative G-V curves from a patch expressing Slo1 D362A:D367A:E399A:Δ894–895 + β1 channels. Blue, control; red, 10 µM PIP2; gray, 10 µM PIP2 + 2 mM Ca^{2+}; green, wash. Similar results were obtained in 10 patches all together (ΔV_{0.5} = −15.7 ± 4.2 mV). (C) Changes in V_{0.5} by PIP2, PIP2 + Ca^{2+}, and wash. Error bars represent mean ± SEM.
Figure S5. Scaled currents through Slo1 complexes with auxiliary subunits with point mutations. (A) Scaled currents at 200 (top) and −200 mV (bottom) from Slo1 + β1 with β1-to-β2 point mutations (red). For each mutant, the currents obtained from Slo1 alone (black) and Slo1 + wild-type β1 (blue) are shown for comparison. (B) Scaled currents at 200 (top) and −200 mV (bottom) in Slo1 + β1 with mutations at position 11. For each mutant, the currents obtained from Slo1 alone (black) and Slo1 + wild-type β1 (blue) are shown for comparison. (C) Scaled currents at 200 (top) and −200 mV (bottom) in Slo1 + β2 Δ2–32 with β2-to-β1 point mutations (red). For comparison, scaled currents from Slo1 alone (black) and Slo1+ wild-type β2 Δ2–32 (blue) are shown. (D) Scaled currents at 200 (top) and −200 mV (bottom) in Slo1 + β4 with β4-to-β1 point mutations (red). For comparison, scaled currents from Slo1 alone (black) and Slo1 + wild-type β4 (blue) are shown. The sweep width represents mean ± SEM; \( n = 4–12 \). All results were obtained without Ca²⁺.
Figure S6. PIP2 enhances currents through divalent-insensitive Slo1+β1 channels. (A) Representative currents through Ca2+- and Mg2+-insensitive Slo1 D362A:D367A:E399A:Δ894–895 + β1 channels before and after the application of 10 µM PIP2. (B) G-V curves in Slo1 D362A:D367A: E399A:Δ894–895 + β1 channels before (blue) and after (red) the application of 10 µM PIP2. The curves represent Boltzmann fits with $V_{0.5} = 201.8 \pm 2.1$ mV and $Q_{app} = 1.03 \pm 0.05$ (Control), and $V_{0.5} = 148.5 \pm 1.4$ mV and $Q_{app} = 1.02 \pm 0.05$ (10 µM PIP2); $n = 14$. 