Direct Regulation of BK Channels by Phosphatidylinositol 4,5-Bisphosphate as a Novel Signaling Pathway

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Large conductance, calcium- and voltage-gated potassium (BK) channels are ubiquitous and critical for neuronal function, immunity, and smooth muscle contractility. BK channels are thought to be regulated by phosphatidylinositol 4,5-bisphosphate (PIP₂) only through phospholipase C (PLC)–generated PIP₂ metabolites that target Ca²⁺ stores and protein kinase C and, eventually, the BK channel. Here, we report that PIP₂ activates BK channels independently of PIP₂ metabolites. PIP₂ enhances Ca²⁺-driven gating and alters both open and closed channel distributions without affecting voltage gating and unitary conductance. Recovery from activation was strongly dependent on PIP₂ acyl chain length, with channels exposed to water-soluble diC₄ and diC₈ showing much faster recovery than those exposed to PIP₂ (diC₁₆). The PIP₂–channel interaction requires negative charge and the inositol moiety in the phospholipid headgroup, and the sequence RKK in the S₆–S₇ cytosolic linker of the BK channel-forming (cbv1) subunit. PIP₂-induced activation is drastically potentiated by accessory β₁ (but not β₃) channel subunits. Moreover, PIP₂ robustly activates BK channels in vascular myocytes, where β₁ subunits are abundantly expressed, but not in skeletal myocytes, where these subunits are barely detectable. These data demonstrate that the final PIP₂ effect is determined by channel accessory subunits, and such mechanism is subunit specific. In HEK293 cells, co-transfection of cbv1+β₁ and PI4-kinaseIα robustly activates BK channels, suggesting a role for endogenous PIP₂ in modulating channel activity. Indeed, in membrane patches excised from vascular myocytes, BK channel activity runs down and Mg-ATP recovers it, this recovery being abolished by PIP₂ antibodies applied to the cytosolic membrane surface. Moreover, in intact arterial myocytes under physiological conditions, PLC inhibition on top of blockade of downstream signaling leads to drastic BK channel activation. Finally, pharmacological treatment that raises PIP₂ levels and activates BK channels dilates de-endothelized arteries that regulate cerebral blood flow. These data indicate that endogenous PIP₂ directly activates vascular myocyte BK channels to control vascular tone.

INTRODUCTION

Blood circulation depends on the myogenic tone of small, resistance-size arteries (Meininger and Davis, 1992). While myogenic tone is regulated by endothelial, neuronal, and circulating factors, it is ultimately determined by the activity of ion channels and signaling molecules in the myocyte itself (Faraci and Heistad, 1998). Tone is increased by a rise in overall cytosolic calcium (Ca²⁺) in the myocyte, which can be achieved by Ca²⁺ influx via depolarization-activated Ca²⁺ channels in the cell membrane and/or Ca²⁺ release from intracellular stores (Jaggar, 2001). Depolarization and increases in Ca²⁺ lead to activation of large-conductance, Ca²⁺/voltage-gated K⁺ (BK) channels, which generate outward currents that tend to hyperpolarize the membrane and thus close voltage-gated Ca²⁺ channels. Therefore, BK channel activation limits voltage-dependent Ca²⁺ entry and myocyte contraction (Brayden and Nelson, 1992; Jaggar et al., 2005).

Phosphatidylinositol 4,5-bisphosphate (PIP₂) plays a key role as an intermediate molecule in many receptor-mediated signaling pathways, including those regulating myocyte contraction (Tolloczko et al., 2002). PIP₂ hydrolysis by PLC renders 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG) (Nahorski et al., 1994). IP₃ mobilizes sarcoplasmic Ca²⁺, while DAG activates PKC. Mobilized Ca²⁺ and activated PKC eventually regulate myocyte BK channel activity (Jaggar et al., 1998; Jaggar, 2001). It is thought that, by producing IP₃ and DAG, PIP₂ indirectly modulates BK channels, and thus myocyte contraction. However, PIP₂ also acts as a signaling molecule.

Abbreviations used in this paper: BK, Ca²⁺/voltage-gated K⁺; C/A, cell-attached; DAG, diacylglycerol; DOG, 1,2-dioctanoyl-sn-glycerol; FA, fatty acids; GPCR, Gq-coupled receptor; HEDTA, 1.6 N-(2-hydroxyethyl)-ethylenediamine-triacetic acid; I/O, inside-out; IP₃, 1,4,5-trisphosphate; OA, okadaic acid; O/O, outside-out; PC, 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine; PIP₂, phosphatidylinositol 4,5-bisphosphate; PPI, phosphoinositide; PS, 1,2-dipalmitoyl-sn-glycerol-3-phospho-L-serine; PSS, physiological saline solution; RT, reverse transcription; SR, sarcoplasmic reticulum; 4-AP, 4-aminopyridine.
on its own through direct interaction with target proteins. In particular, PIP₂ directly modulates the activity of ion channels and transporters (Hilgemann and Ball, 1996; Fan and Makielksi, 1997; Rummels et al., 2002; Rohács et al., 2003; Chemin et al., 2005; Süh and Hille, 2005; Brauchi et al., 2007; Hilgemann, 2007; Rohács, 2007; Voets and Nilius, 2007). In spite of the key roles of PIP₂ and BK channels in cell excitability and signaling, it remains unknown whether PIP₂ can directly modulate BK channel function.

Here, we demonstrate that PIP₂ directly (i.e., independently of PIP₂ metabolites and downstream signaling) increases BK channel steady-state activity, the pore-forming (cbv1) subunit being sufficient to sense the phosphoinositide (PIP). The cbv1–PIP₂ interaction requires recognition of negative charges and the inositol moiety in the PIP₂ headgroup by a channel sequence that meets major criteria for a PIP₂ binding site. This interaction results in a drastic increase in the channel’s apparent Ca²⁺ sensitiv-
ity, with changes in both open and closed time distributions. PIP₂ action on cbv1 channels is drastically amplified by coexpression of the smooth muscle–abundant, channel accessory β₁ subunit but not other (e.g., β₄), subunit. PIP₂ robustly activates native BK channels in vascular myocytes where β₁ is highly expressed, but not in skeletal myocytes, where β₁ is barely detected. Using intact vascular myocytes under physiological conditions of Ca²⁺ and voltage, we demonstrate that endogenous PIP₂ plays a role in activating BK channels via the direct mechanism. Furthermore, manipulation of endogenous PIP₂ levels dilates pressurized, resistance-size cerebral arteries, an effect that is prevented by selective BK channel block.

**MATERIALS AND METHODS**

**Cerebral Artery Diameter Measurement and Myocyte Isolation**

Sprague-Dawley rats (250 g) were decapitated, and middle cerebral and basilar arteries were isolated. Following endothelium removal and artery pressurization (Liu et al., 2004), vessels were extraluminally perfused with physiological saline solution (Liu et al., 2004) at 3.75 ml/min using a peristaltic pump (Rainin). Drug stock solutions (see below) were diluted in PSS to final concentration. Diameter changes were determined with IonWizard 4.4 (IonOptics).

Single myocytes were isolated from cerebral arteries following procedures already described (Liu et al., 2004; Bukiya et al., 2007). Skeletal muscle fibers were prepared using slight modifications to methods described elsewhere (McKellen et al., 1994). In brief, flexor digitorum brevis muscle was dissected from adult Sprague-Dawley rats and incubated in 0.3% collagenase (Type 1) in Ringer solution (in mM): 146.3 NaCl, 4.75 KCl, 1 CaCl₂, 0.95 Na₂HPO₄, 0.5 MgCl₂, 9.5 HEPES, adjusted to pH 7.4 with NaOH. Muscles were incubated in this solution at 4°C for 30 min and switched to 37°C for 90 min. Single fibers were isolated in Ringer solution without collagenase by triturating the tissue with fire-polished Pasteur pipettes. The isolated fibers were then placed in a solution containing (in mM) 139 KCl, 5 EGTA, 10 HEPES, adjusted to pH 7.4 with KOH. In this solution, sarcolemmal vesicles formed on the surface of the muscle fibers.

**Perforated-Patch Experiments on Vascular Myocytes.** The pipette solution contained (in mM) 110 K-aspartate, 30 KCl, 10 NaCl, 1 MgCl₂, 10 HEPES, and 0.05 EGTA, with pH adjusted to 7.2 by adding KOH. The perforated-patch configuration was achieved by adding amphoterin B dissolved in DMSO into pipette solution at a concentration of 250 μg/ml. Myocytes were bathed in HEPES-buffered physiological saline (PSS). PSS had the following composition (in mM): 134 NaCl, 6 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose, with pH adjusted to 7.4 by adding NaOH.

**Excised Patch Recordings from Vascular Myocytes.** For inside-out (I/O) recordings, the electrodes were filled with (in mM) 130 KCl, 5.22 CaCl₂, 2.28 MgCl₂, 15 HEPES, 5 EGTA, and 1.6 N(2-hydroxyethyl)-ethylenediamine-triacetic acid (HEDTA), with pH

**Mutagenesis and cRNA Injection**

cDNA encoding cbv1 was cloned using PCR and reverse transcription (RT) from total RNA of myocytes freshly isolated from rat small cerebral arteries (Quinn et al., 2003; Jaggar et al., 2005). The pOX vector and full-length cDNAs coding for β₁ and β₄ were gifts from A. Wei (Washington University at St. Louis, St. Louis, MO), M. Garcia (Merck Research Laboratories, Rahway, NJ), and L. Toro (University of California at Los Angeles, Los Angeles, CA). We used Quikchange (Stratagene) to mutate RKK in the cbv1 S6–S7 linker. Sequencing was conducted at the University of Tennessee Molecular Research Center. cDNA coding for cbv1 was cleaved with BamHI (Invitrogen) and Xhol (Promega) and inserted into pOX. pOXB-cbv1 and pOXB-RKcbv1AAA were linearized with NotI and SacII (Promega) and transcribed in vitro using T3. PBS×MXT-K293cbv1A was linearized by SalI and transcribed in vitro using T3. BK β₁ cDNA inserted into pCI-Neo was linearized with NotI and transcribed in vitro using T7. BK β₁ cDNA inserted into pOx was linearized with NotI and transcribed using T3. The mMessage-mMachine kit (Ambion) was used for transcription.

Oocytes were removed from Xenopus laevis and prepared as previously described (Dopico et al., 1998). cRNA was dissolved in DEPC-treated water at 5 (cbv1) and 15 (β₁ or β₄) ng/μl. 1-μl aliquots were stored at −70°C. Cbl1 cRNA was injected alone (2.5 ng/μl) or co-injected with β₁ or β₄ (7.5 ng/μl) cRNAs, giving molar ratios 26:1 (β₁α) (Bukiya et al., 2007). Expression of the mutated cbv1 was lower than that of wild type (wt); thus, cRNA was increased to 3 μg/μl for a total volume of 23 nl. After cRNA injection, oocytes were prepared for patch-clamping as previously described (Dopico et al., 1998).

**Cell Culture and Transfection**

HEK-293 cells were transfected with pcDNA3 vector-cbv1 cDNA and pCI-Neo vector-β₁ cDNA with or without pCMV5 vector-P4KIIa cDNA. Transfection was performed with Lipofectamine 2000 (Invitrogen).

**Electrophysiology**

Currents were acquired using an EPC8 amplifier (List), low-passed at 1 kHz with an 8-pole Bessel filter (Frequency Devices), and digitized at 10 kHz using 1320 Digidata/pClamp8 (Molecular Devices). Data from single channel patches for dwell-time analysis were acquired at 7 kHz and digitized at 35 kHz. Patch pipettes were prepared as described elsewhere (Dopico et al., 1998). Experiments were performed at room temperature. Solutions were made with deionized (18 MΩ-cm) water and high-grade purity salts. Free Ca²⁺ concentrations were calculated using Max Chelator Sliders (C. Patton, Stanford University, Stanford, CA) and validated experimentally (Dopico, 2003). A variety of solutions were used, as follows.

For inside-out (I/O) recordings, the electrodes were filled with (in mM) 110 K-aspartate, 30 KCl, 10 NaCl, 1 MgCl₂, 10 HEPES, and 0.05 EGTA, with pH adjusted to 7.2 by adding KOH. The perforated-patch configuration was achieved by adding amphoterin B dissolved in DMSO into pipette solution at a concentration of 250 μg/ml. Myocytes were bathed in HEPES-buffered physiological saline (PSS). PSS had the following composition (in mM): 134 NaCl, 6 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose, with pH adjusted to 7.4 by adding NaOH.

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adjusted to 7.4 by adding KOH and a free \([\text{Ca}^{2+}] \approx 0.3 \mu \text{M}\). For outside-out (O/O) recordings, with okadaic acid (OA, 2 nM) (Lin et al., 2005). PIP\(_2\) monoclonal the BK channel was reactivated with Mg-ATP (0.5 mM) together immediately after excision, I/O currents were recorded at 0, 3, 10, and 30 min. After 30 min (maximal rundown; Lin et al., 2005), the BK channel was reactivated with Mg-ATP (0.5 mM) together with okadaic acid (OA, 2 nM) (Lin et al., 2005). PIP\(_2\) monoclonal antibodies (1:1,000, Assay Designs) were applied to the cytosolic side of the membrane.

**Skeletal Muscle BK Channel Recordings.** Membrane patches were excised from isolated skeletal muscle fibers, and BK currents were recorded in the I/O configuration by using techniques similar to those described for vascular myocyte I/O recordings. The bath solution, however, contained (in mM) 130 KCl, 2.28 MgCl\(_2\), 15 HEPES, 5 EGTA, and 1.6 HEDTA, with pH adjusted to 7.4 by adding KOH; free \([\text{Ca}^{2+}] \approx 3 \mu \text{M}\). Immediately after excision, I/O currents were recorded at 0, 3, 10, and 30 min. After 30 min (maximal rundown; Lin et al., 2005), the BK channel was reactivated with Mg-ATP (0.5 mM) together with okadaic acid (OA, 2 nM) (Lin et al., 2005). PIP\(_2\) monoclonal antibodies (1:1,000, Assay Designs) were applied to the cytosolic side of the membrane.

**Oocyte Recordings.** Oocytes were isolated from *X. laevis* and treated for patch-clamp recordings as mentioned in the text and described in detail elsewhere (Dopico et al., 1998). Recordings were performed in the I/O configuration; the electrode and bath solutions had compositions similar to the electrode and bath solutions used in myocyte experiments (see above), except that K-glucuronate replaced KCl to avoid contaminating recordings with endogenous Ca\(^{2+}\)-activated Cl\(^-\) channel activity (Dopico et al., 1998). In this series of experiments, bath \([\text{Ca}^{2+}]\) was set to 0.3 or 10 \(\mu \text{M}\) by changing the amount of CaCl\(_2\) and EGTA buffer.

**Experiments on Transfected HEK Cells.** Cells were transfected and cultured as described above. Recordings were obtained in the I/O configuration. The bath solution contained (in mM) 5 Na\(^+\)-glucuronate, 140 K\(^+\)-glucuronate, 1 MgCl\(_2\), 15 HEPES, 0–4 HEDTA, 0–4 EGTA, and 0.45–2.2 CaCl\(_2\), pH adjusted to 7.35 with KOH. The concentrations of CaCl\(_2\), EGTA, and HEDTA were adjusted to obtain the desired concentrations of free metal as described above. The electrode solution contained (in mM) 140 K\(^+\)-glucuronate, 1 MgCl\(_2\), 22 mM CaCl\(_2\), 15 HEPES, 4 HEDTA, and 4 EGTA. Cells were washed for 30 min in 2.2 mM Ca\(^{2+}\) bath solution before recordings. Single channel records were obtained as explained above for oocytes and myocytes.

**Voltage Protocols and Data Analysis.** For perforated-patch recordings in myocytes, the membrane was held at −80 mV, and total outward currents were evoked by 0.2-s, 20-mV depolarizing steps from −60 to 100 mV; leak currents were determined using a P/4 protocol. Peak current amplitude was determined 0.14–0.19 s after the start of the pulse and obtained after digital subtraction of leak from total current. For macroscopic excise patch recordings in oocytes, the membrane was held at 0 mV, and total outward currents were evoked by 0.2-s, 10-mV depolarizing steps from −100 to 200 mV. Peak current amplitude was determined 0.14–0.19 s after the start of the pulse. As index of channel steady-state activity, we used the product of the number of channels present in the membrane patch (N) and the channel open probability (P\(_o\)). N\(_p\) was calculated from all-points amplitude histograms (Dopico et al., 1998). At the beginning of each experiment, N\(_p\) was determined from ≥5 min to ensure that changes in activity at the time of reagent application were due to the reagent itself and not to nonstationary N\(_p\). N\(_p\) under a given condition was obtained from >3 min of continuous recording. Dwell time analysis was conducted as previously described (Dopico et al., 1998; Crowley et al., 2003). Channel mean open time (\(t_o\)) in multichannel patches of unknown N was obtained from \(t_o = N_p \times T/\theta_{o}\) where \(\theta_{o}\) is the number of channel openings during several minutes (T) of continuous current recording under each condition (Fenwick et al., 1982; Dopico et al., 1998). Data and idealized records were analyzed using pClamp 9.2 (Molecular Devices) as described elsewhere (Dopico et al., 1998) and plotted and fitted using Origin 6.1 (Origin Laboratory).

**Compounds and their Application**

Stock solutions of anionic phospholipids (PS [synthetic], PI [synthetic], Echelon Biosciences], PI5P [synthetic], PIP\(_2\) [diC16, synthetic, Calbiochem and Sigma-Aldrich], PIP\(_2\) [synthetic]) were made in ultrapure distilled water at a lipid concentration of 10 \(\mu \text{M}\) by sonication on ice for 30 min immediately before the experiment. PC (semisynthetic), a switchole phospholipid, was first dissolved in pure DMSO at a concentration of 2 \(\mu \text{M}\) and then mixed and sonicated for 30 min in recording solution to obtain a final lipid concentration of 10 \(\mu \text{M}\). Dibutanyol and dioctanoyl PIP\(_2\) (synthetic, Sigma-Aldrich) (diC4 and diC8) were diluted in ultrapure distilled water at a lipid concentration of 10 \(\mu \text{M}\). The lipid-containing solutions were applied to the cytosolic side of the patch membrane immediately after the dispersal procedure. For the O/O and whole-cell recordings, lipid-containing solutions were applied to the external side of the membrane. For the C/A recordings, lipid-containing solutions were applied to the extracellular, extrapatch surface of the cell.

Poly-L-lysine was dissolved in high-purity deionized water (50 mg/ml stock), further diluted in bath solution to 100 \(\mu \text{g}\)/ml, and applied to the cytosolic side of I/O patches. 1,2-dioctanoyl-sn-glycerol (DOG) was dissolved in DMSO (1 mg/ml stock), further diluted in bath solution to 2 \(\mu \text{M}\), and applied to the cytosolic side of I/O patches. Before recording changes in channel activity evoked by a given compound, control recordings were obtained when a steady-state perfusion was achieved, which typically took 5–10 min.

For the perforated-patch recordings, agent-containing solutions were applied to the extracellular, extrapatch surface of the cell. Ro 31-8220 (Biomol Research Laboratories) was reconstituted in DMSO stock (10 \(\mu \text{g}\)/ml) and diluted in bath solution to a final concentration of 2 \(\mu \text{M}\). Thapsigargin was reconstituted in DMSO stock (10 mM) and diluted in bath solution to a final concentration of 200 \(\mu \text{M}\). Paulline was dissolved in DMSO as a 25 mM stock and further diluted in bath solution to a final concentration of 500 \(\mu \text{M}\). Wortmannin (Stressgen Bioreagents) was reconstituted in DMSO stock (50 mg/ml) and diluted in bath solution to a final concentration of 5 \(\mu \text{M}\). U73122 (Biomol Research Laboratories) was reconstituted in DMSO stock (4 mM) and diluted to a final concentration of 5–25 \(\mu \text{M}\). 4-aminoypyridine (4-AP) was dissolved in high-purity deionized water as a 0.8 \(\mu \text{M}\) stock and further diluted in bath solution to a final concentration of 5 \(\mu \text{M}\). 2-[[3-(trifluoromethyl)phenyl] amino]pyridine-3-carboxylic acid (niflumic acid) was dissolved in acetone as a 0.2 \(\mu \text{M}\) stock and further diluted in bath solution to a final concentration of 100 \(\mu \text{M}\).

Compounds applied to pressurized vessels were diluted to make stock solutions as described above and then further diluted in PSS to final concentration. Unless otherwise stated, all compounds were purchased from Sigma-Aldrich.
PIP$_2$ Directly Activates BK Channels

**RESULTS**

**PIP$_2$ Activates BK Channels in Cerebral Artery Myocytes**

We first applied PIP$_2$ to the cytosolic side of I/O patches excised from freshly isolated myocytes and determined responses in channel steady-state activity (NP$_o$; see Materials and methods). Studies were conducted with Ca$^{2+}$i = 0.3 μM, which is found in cerebral artery myocytes (Knot and Nelson, 1998; Pérez et al., 2001). The membrane potential was held positive to evoke an easily measurable NP$_o$. PIP$_2$ at levels found in plasma membranes (10 μM; McLaughlin and Murray, 2005) increased NP$_o$ (5/5 cells) (Fig. 1 A), reaching 2,831 ± 202% of control. In submicromolar concentrations of Ca$^{2+}$i, PIP$_2$-induced increase of BK NP$_o$ was sustained, persisting ∼30 min after PIP$_2$ application (Fig. 1 B) and returning to pre-PIP$_2$ values after washout in bath solution for >30 min (Fig. 1 A). These data suggest that the increase in BK NP$_o$ is due to PIP$_2$ itself, instead of PIP$_2$ active metabolites.

The more water-soluble diC$_4$ and diC$_8$ analogues also readily increased BK NP$_o$ ($n$ = 4; V = +40 mV), with NP$_o$ readily turning to preanalogue values with wash in bath solution. The diC$_4$ and diC$_8$ effect, however, differed from that of PIP$_2$ in two aspects. First, the potentiation of channel activity was much more robust for PIP$_2$ than those caused by the two more soluble analogues: 2,831 ± 202, 308 ± 56, and 230 ± 34% of control for PIP$_2$, diC$_8$, and diC$_4$, respectively. As discussed with inward rectifier K$^+$ channels (Rohács et al., 1999; Cho et al., 2006), the increased effectiveness of PIP$_2$ in potentiating BK likely reflects the increased partition of this hydrophobic analogue in the lipid environment and, eventually, more effective loading of the cell membrane with increased access to the channel target. Second, recovery from potentiation was much faster for diC$_4$ and diC$_8$ (Fig. 1 C). Conceivably, the fast relaxation of these analogues reflects elimination of bound diC$_4$/diC$_8$ monomers from a binding site(s) that is readily accessible from the aqueous phase. In contrast to diC$_4$ and diC$_8$, lipids with longer side chains such as PIP$_2$, are not only in monomeric but also (and mainly) in micellar

**Online Supplemental Material**

The online supplemental material (available at http://www.jgp.org/cgi/content/full/jgp.200709913/DC1) contains one figure showing both representative unitary current recordings and averaged channel activity data in response to application of 10 μM PIP$_3$ to the cytosolic side of inside-out patches from *Xenopus laevis* oocytes that express wt cbv1, RKKcbv1AAA, or K239cbv1A. Results show that the PIP$_3$ responses in the K239A mutant are similar to those in wt cbv1, while RKKcbv1AAA responses are significantly blunted, indicating that the RKKcbv1AAA mutation in the cbv1 S6–S7 linker distinctly reduces PIP$_3$ activation of cbv1 channels.
Because PIP$_2$ effects on NP$_o$ were recorded in cell-free patches (even >20 min after excision) in a highly buffered Ca$^{2+}$ solution containing no nucleotides, it is unlikely that cytosolic messengers mediate PIP$_2$ action. Rather, PIP$_2$ targets the BK channel itself, its proteolipid microenvironment, or a lipid–protein interface. In contrast to I/O results, PIP$_2$ failed to consistently increase NP$_o$ when applied to the extra-patch membrane of C/A patches (Fig. 1D). This is consistent with the difficulty that a charged molecule (charge ≈ $-\frac{\mu C}{mL}$) may have in accessing a target located in the membrane within the pipette. The PIP$_2$ effect was also mild and inconsistent when the lipid was applied to the extracellular side of O/O patches (Fig. 1D). The contrast between I/O and C/A or O/O results indicates that PIP$_2$ accesses its site of action most effectively from the form in the aqueous phase (Flanagan et al., 1997; Huang et al., 1998). Thus, PIP$_2$ micelles can incorporate into the bilayer to form mixed micelles. Release of these micelles from the membrane should take times much longer than those corresponding to bound-monomer dissociation from a target site, resulting in slower channel recovery from activation (Rohács et al., 1999).

The increased NP$_o$ caused by PIP$_2$ application was not accompanied by any noticeable change in unitary current amplitude (Fig. 1A). Slope unitary conductance remained constant in the presence of PIP$_2$ when evaluated across a voltage range at which the current was ohmic (−60 to 40 mV in 1 mM Mg$^{2+}$, and symmetric 130 mM K$: 243$ vs. 251 pS, control and PIP$_2$). Thus, within this voltage range, any PIP$_2$ modification of macroscopic current should be attributed to PIP$_2$ action on NP$_o$.
Acyl chains. Dipalmitoyl chains were chosen because their length mimics that of phospholipid acyl chains prevalent in natural membranes. When applied to the intracellular side of I/O patches, all phosphoinositides readily increased NP o (Fig. 2 A). Moreover, channel activation correlated positively with the number of negative charges in the phospholipid headgroup: NP o = 957, 1801%, 2831%, and 3629% of controls for D (+)-sn-1,2-dipalmitoyl-glyceryl, 3-O-phospho linked (PI) (1), 1,2-dipalmitoyl-l-α-phosphatidyl-d-myoinositol 5-monophosphate (PIP5) (2), PIP2 (3), and 1,2-dipalmitoylphosphatidylinositol 3,4,5-trisphosphate (PIP3) (4) (Fig. 2 B).

Among all phosphoinositides tested, PIP3 showed the highest effectiveness, whether evaluated in myocyte native cytosolic side of the membrane, where PIP2 is naturally predominant (Laux et al., 2000).

Structural Determinants of PIP2 Action
Negative charges and the position of the phosphates in the inositol ring are important for phosphoinositide interaction with Kir channels (Suh and Hille, 2005). In addition, the BK channel displays higher P o when reconstituted in lipid bilayers that include negatively charged phospholipids (Park et al., 2003). Thus, we next probed phospholipids having different negative charges in their headgroups. Because PIP2 chain length modified the magnitude and time course of BK channel activation (see previous section), we used lipid species having the same acyl chains. Dipalmitoyl chains were chosen because their length mimics that of phospholipid acyl chains prevalent in natural membranes. When applied to the intracellular side of I/O patches, all phosphoinositides readily increased NP o (Fig. 2 A). Moreover, channel activation correlated positively with the number of negative charges in the phospholipid headgroup: NP o = 957, 1801%, 2831%, and 3629% of controls for D (+)-sn-1,2-dipalmitoyl-glyceryl, 3-O-phospho linked (PI) (1), 1,2-dipalmitoyl-l-α-phosphatidyl-d-myoinositol 5-monophosphate (PIP5) (2), PIP2 (3), and 1,2-dipalmitoylphosphatidylinositol 3,4,5-trisphosphate (PIP3) (4) (Fig. 2 B).

Among all phosphoinositides tested, PIP3 showed the highest effectiveness, whether evaluated in myocyte native
BK channels (NPo = ~3,500% of control; Fig. 2 B) or cbv1 expressed in X. oocytes (NPo = ~900% of control). From multichannel patches, we determined thatPIP3 raised the channel mean open time (tO) from 0.39 to 0.51 ms. The increment in tO (+31%) cannot account solely for the PIP3-induced increase in PO (~900%). Therefore, the drastic increase in PO in response to PIP3 must be attributed to a combination of mild increase in tO and robust increase in frequency of channel openings (i.e., a decrease in channel mean closed time; Dopico et al., 1998), the latter being evident in the traces shown in Fig. S1 A.

Adding the polycationic PIP2 scavenger poly-l-lysine to the bath solution (0.1 mg/ml) (Quinn et al., 2003) significantly reduced PIP2 action: NO in PIP2 reached only 975% of control in the presence of poly-l-lysine, in contrast to the 2,831% of control obtained in the same patch when recorded in poly-l-lysine-free solution (Fig. 2 C). Poly-l-lysine itself, however, usually failed to readily (<3 min) modify NPo (unpublished data). Thus, poly-l-lysine’s blunting of PIP2 action appears not to result from opposite modulation of NPo by the polycation and the negatively charged lipid. After 3 min of poly-l-lysine application, BK NPo did decrease significantly (−35 ± 1.4%; n = 3). Collectively, results with poly-l-lysine are interpreted as the polycation scavenging PIP2 via salt bridges formed between the positive charges of the former and the negative headgroup of the latter. Finally, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (PC), a phospholipid with no net charge at physiological pH (i.e., our recording conditions), barely increased NPo (Fig. 2 E). Collectively, data indicate that the amount of negative charge in the phospholipid headgroup is a key determinant for PIP2 and analogues to activate arterial myocyte BK channels.

We next examined whether the specific structure of the headgroup contributes to phosphoinositide action on the BK channel by probing PI vs. 1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine (PS). Both phospholipids carry the same net charge (−1) under our recording conditions, but differ in their headgroup base (inositol vs. serine). PI increased NPo to 957% of control, which is substantially greater than that caused by PS (NPo = 356% of control) (P < 0.001) (Fig. 2 E). Therefore, added to the negative charge, the inositol moiety favors channel activation. This structural specificity is consistent with the idea that phosphoinositides target a defined protein site.

Cvb1 Is Sufficient for PIP2 Action, which Is Drastically Amplified by β3, but Not β2, Subunits

Cerebrovascular myocyte BK channels consist of pore-forming α (cbv1, encoded by KCNN1) and accessory β3 (KCNNB1) subunits (Ortiz et al., 2002; Jaggar et al., 2005; Liu, J., P. Liu, M. Asuncion-Chin, and A. Dopico. 2005. Soc. Neurosci. Abstr. Online. 960:913) (Fig. 3 A). After cbv1 expression in Xenopus oocytes, application of PIP2 to the cytosolic side of I/O patches consistently activated cbv1 channels (Fig. 3 B), NPo reaching 590 ± 15% of control. This indicates that cbv1 and its immediate lipid environment are sufficient for PIP2 activation of BK channels. Having established that headgroup negative charge plays a critical role in this action, we probed whether positively charged residues in cbv1 could recognize the negatively charged PIP2. Alanine substitution of positive residues clustered at the bottom of the pore-forming M2 domain of Kir6.2 channels attenuates PIP2 modulation (Shyng et al., 2000). Similarly, positive residues clustered at the bottom of the pore-forming S6 segment of KCNQ1 are thought to contribute to PIP2 action on KCNQ1/KCNE1 channels (Loussouarn et al., 2003). After identifying a cluster of positive residues in cbv1, we probed PIP2 in cbv1 where AAA substituted for RKK in the S6–S7 cytosolic linker (Fig. 3 A).

In I/O patches, RKKcbv1AAA currents were indistinguishable from those mediated by cbv1 (Fig. 3 C). In contrast to wt cbv1, PIP2 applied to the cytosolic side of I/O patches expressing RKKcbv1AAA barely shifted the G/Gmax–voltage curve (Fig. 3 C). PIP2 differential action on wt cbv1 vs. RKKcbv1AAA was also evident from

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**Figure 4.** PIP2 action on native BK channels in isolated skeletal muscle myocytes. (A) Unitary currents from an I/O patch obtained before (top) and after (bottom) a 5-min bath application of 10 μM PIP2, show that the phosphoinositide causes an increase in BK NPo, that is marked reduced when compared with that evoked by PIP2 in vascular myocyte BK channels (Fig. 1, A and C). Arrowheads, baseline; upward deflections, channel openings. (B) Averaged PIP2 responses of native skeletal muscle BK channels; *, P < 0.05; n = 4; V = 40 mV, Ca2+ = 10 μM.
channel activity) (Fig. 3 C). Under conditions identical to those used in the studies of PIP$_2$ action on wt cbv1 and RKKcbv1AAA channels, PIP$_2$ potentiated K239A-mediated current when studied at both macroscopic (Fig. 3 C) and single-channel levels (Fig. 3 D). Moreover, the magnitude of K239A channel activation in response to PIP$_2$ was identical to that observed with wt cbv1 (Fig. 3 D). Therefore, PIP$_2$ activation of cbv1 does not involve any positive amino acid residue that can be found in cbv1 cytosolic loops. Rather, the S6–S7 RKK and its flanking sequence, which meet criteria for a PIP$_2$ binding site (see Discussion), appear specifically involved. As found with PIP$_2$, PIP$_3$ action was also significantly blunted in RKKcbv1AAA, yet similar in wt cbv1 and the K239A mutant (Fig. S1), suggesting a common site(s) of action for PIP$_2$ and PIP$_3$.

Notably, PIP$_2$ action on cbv1 expressed in oocytes was significantly smaller than that observed with native channels in myocytes (Fig. 3 D vs. Fig. 2 D). Because cbv1+$\beta_1$ constitutes the cerebral artery myocyte BK, we next explored PIP$_2$ action on cbv1+$\beta_1$ expressed in single-channel data: PIP$_2$ caused a mild increase in RK-Kcbv1AAA NP$_o$, which was drastically smaller than that in wt cbv1 (Fig. 3 D). Furthermore, exposing the mutant to increased PIP$_2$ (30 μM) raised NP$_o$ to ~350% of control (n = 4), which is barely different from the response evoked by 10 μM PIP$_2$ in the mutant and significantly smaller than the response in wt cbv1. Therefore, the sequence RKK in the cbv1 S6–S7 linker is involved in PIP$_2$ activation of the BK channel.

To determine whether PIP$_2$ activation of cbv1 channels specifically depends on the S6–S7 RKK sequence or, rather, can also depend on positive amino acids located in other cbv1 cytosolic loops, we made the construct K239cbv1A. K239 is located in the S4–S5 cytosolic loop and is thus readily accessible to PIP$_2$ negative charges when the phosphoinositide is applied to the cytosolic membrane leaflet. After expression in Xenopus laevis oocytes, K239cbv1A rendered channel current events characteristic of BK (slo1) channels, such as high unitary conductance for K$^+$ (Fig. S1 A) and voltage dependence of channel gating (12.9 mV/e-fold change in Ca$^{2+}$-driven gating, resulting in modifications of both open and closed times. (A) PIP$_2$ activation of cbv1 is negligible at zero nominal Ca$^{2+}$ and reaches a maximum at 10 μM Ca$^{2+}$$; (a) zero Ca$^{2+}$ vs. 0.3 μM Ca$^{2+}$: P < 0.001; (b) zero Ca$^{2+}$ vs. 10 μM Ca$^{2+}$: P < 0.001; (c) zero Ca$^{2+}$ vs. 100 μM Ca$^{2+}$: P < 0.001; (d) 0.3 μM Ca$^{2+}$ vs. 10 μM Ca$^{2+}$: P < 0.001; (e) 0.3 μM Ca$^{2+}$ vs. 100 μM Ca$^{2+}$: P < 0.001; n = 3–12. (B) Single channel records in the absence (top) and presence (bottom) of 10 μM PIP$_2$ show that the lipid increases P$_o$ from 0.06 to 0.45 (616% of control). Records were low-passed at 7 kHz and digitized at 35 kHz. Upward deflections: channel openings. (C) Open and (D) closed time distributions in control (top) and PIP$_2$ (bottom) from records shown in B. Each lifet ime constant (τ) is shown in milliseconds, with its contribution to the total fit in parentheses. Each component of a fit is shown with a dotted line, and the composite fit is shown with a solid line; 40 mV; Ca$^{2+}$ = 0.3 μM.
Xenopus oocytes. The presence of functional β₁ was confirmed by current characteristics, or a Pₒ increase with bath application of 10 μM 17β-estradiol to O/O patches (Bukiya et al., 2007). PIP₂ activation of cbv1 was consistently enhanced when β₁ was coexpressed (Fig. 3, E and F). Notably, the PIP₂ increase in cbv1+β₁ Pₒ (2,029 ± 286% of control) (Fig. 3 F) was similar to that in the myocyte native channel (Fig. 1 C). Thus, possible differences in the proteolipid environment around the BK channel complex between frog oocyte and rat myocyte membranes appear not to play a major role in PIP₂ action. Rather, the cbv1+β₁ complex appears sufficient to support channel activation by PIP₂.

To determine whether the amplification of PIP₂ action is selective to the β subunit type that is predominant in smooth muscle (β₁), we tested PIP₂ action on cbv1+β₁ complexes. The presence of functional β₄ was confirmed by current characteristics, including refractoriness to iberiotoxin block (Bukiya et al., 2007). Under conditions identical to those used with cbv1+β₁, PIP₂ action on cbv1 was not amplified by β₁ (Fig. 3, E and F). Because the expression of a given β type shows high tissue specificity (Behrens et al., 2000; Brenner et al., 2000; Orio et al., 2002), the differential PIP₂ action on recombinant channels expressed in oocytes raised the speculation that drastic activation of native BK is restricted to cells expressing high amounts of β₁, such as vascular myocytes. Thus, we probed PIP₂ on native BK channels in another type of myocyte (the skeletal muscle fiber), where β₁ is barely expressed (Behrens et al., 2000; Brenner et al., 2000; Orio et al., 2002). Application of 10 μM PIP₂ to the cytosolic side of I/O patches from rat skeletal muscle fibers consistently caused channel activation (Fig. 4). However, this activation was drastically smaller than that evoked by PIP₂ in native vascular myocyte BK channels (Fig. 2 D, first column). Furthermore, PIP₂ action on native skeletal muscle native channels was indistinguishable from that observed with cbv1 in oocytes and significantly smaller than PIP₂ action on cbv1+β₁ channels (Fig. 3 F). Therefore, PIP₂ action is, indeed, strongest in tissues expressing BK channel complexes that contain β₁ subunits.

### PIP₂ Modifies both Open and Closed Time Distributions

We next determined which PIP₂ actions lead to increased BK channel Pₒ. A hallmark of BK channels is independent gating by voltage and Ca²⁺. Application of PIP₂ to the cytosolic side of I/O patches expressing cbv1 resulted in a parallel leftward shift in the macroscopic current conductance (G/G_max)–voltage curve (Fig. 3 C). Thus, the channel effective valence (z) obtained from these plots was identical in the absence and presence of PIP₂: z = 1.71 ± 0.03 vs. 1.7 ± 0.04, P > 0.5 (Ca²⁺ = 0.3 μM). Therefore, the negatively charged lipid modifies Pₒ without affecting the effective gating charge.

A parallel leftward shift in the G/G_max–voltage relationship can be caused by an increase in the apparent Ca²⁺ sensitivity of the channel (i.e., less Ca²⁺ is needed to obtain a given Pₒ). To determine the Ca²⁺ dependence of PIP₂ action, we probed PIP₂ on cbv1 using solutions containing constant free Mg²⁺ (=0.6 mM) and different, highly buffered Ca²⁺ levels. When the channel was primarily gated by voltage (i.e., zero nominal Ca²⁺), PIP₂ barely modified Pₒ (130% of control; Fig. 5 A). PIP₂-induced potentiation, however, was robust at 0.3 μM Ca²⁺, reaching a maximum at 10 μM Ca²⁺ (Fig. 5 A), which suggests that PIP₂ increases Pₒ by amplifying Ca²⁺-driven gating.

![Figure 6](image-url)  
Endogenous PIP₂ maintains native BK channel activity.  
(A) Time course of vascular myocyte BK Pₒ, from an I/O patch. Intervals indicate time after patch excision. Arrowheads, baseline; upward deflections, channel openings. Bath application of 0.5 mM ATP and 0.1 μM okadaic acid (OA) increases Pₒ × 5.4 times. Subsequent application of PIP₂ monoclonal antibodies (1:1,000) drastically reduces Pₒ. V = 40 mV; Ca²⁺ = 3 μM. (B) Cotransfection of HEK cells with PI4K and cbv1+β₁ channel subunits causes a parallel leftward shift in the activation–voltage plot when compared with currents mediated by cbv1+β₁ alone; n = 5–7.
Data from patches containing one functional channel revealed that PIP₂ increase in P₀ (Fig. 5 B) was similar to the potentiation of NP₀ (Fig. 3 D). Therefore, it appears that PIP₂ action on NP₀, and thus current, is due solely to modification of P₀. The increase in P₀ evoked by PIP₂ was always associated with a robust increase in the frequency of channel bursts (Fig. 5 B). However, the PIP₂ effect on P₀ results from several PIP₂ actions, as revealed by dwell-time distribution analysis. PIP₂ caused a major shift in the open channel population toward longer openings, which resulted in an ~500% increase in channel mean open time (Fig. 5 C). In addition, PIP₂ drastically reduced the duration of the channel long closures, which resulted in increased channel bursting (Fig. 5 B) and a drastic reduction in the channel mean closed time, the latter reaching 3.3% of control (Fig. 5 D). In brief, PIP₂ increases BK P₀ by both stabilization of channel openings and destabilization of channel long closures.

Figure 7. Endogenous PIP₂ activates native BK currents in the presence of blockers of PLC-mediated PIP₂ downstream products. Perforated patch recordings from two (A–E and F–J) freshly isolated cerebral artery myocytes bathed in physiological saline solution (PSS; composition in Materials and methods). Total outward K⁺ currents were recorded in the continuous presence of 5 mM 4-AP and 0.1 mM niflumic acid. Bath application of 2 μM Ro31-8220 and 0.2 μM thapsigargin increases mean outward current by 95% (B vs. A, and G vs. F). Subsequent inhibition of PI3 kinase by 5 nM wortmannin further increases current by 184% from control (C). Inhibition of PLC by 25 μM U73122 drastically increases current (D), likely due to buildup of PIP₂ in the membrane. The current is blocked by 0.3 μM paxilline (E and H), indicating it is mediated by BK channels. Preapplication of paxilline, a selective BK channel blocker, prevents both wortmannin (I) and U73122 (J) actions (n = 5–6).

Regulation of BK Channels by Endogenous PIP₂

After showing that exogenously applied PIP₂ enhances BK P₀, we next determined whether channel activity could be modulated by endogenous PIP₂. As reported with BK channels from sheep basilar artery myocytes (Lin et al., 2003), cerebrovascular BK channel NP₀ continuously ran down after patch excision, reaching ~62% of control after 30 min (Fig. 6 A). BK NP₀ is modulated by protein phosphatases and kinases that remain associated with the excised patch (Lin et al., 2003). Additionally, activation of lipid kinases via Mg-ATP increases membrane PIP₂ levels and thus modulates BK channel activity (Huang et al., 1998). To begin to test whether endogenous PIP₂ contributes to regulating BK channel activity in the native membrane. Finally, cotransfection of HEK293 cells with cbv1+ and PI4kinaseII resulted in robust potentiation of NP₀, likely due to buildup of PIP₂ in the membrane. The current is blocked by 0.3 μM paxilline (E and H), indicating it is mediated by BK channels. Preapplication of paxilline, a selective BK channel blocker, prevents both wortmannin (I) and U73122 (J) actions (n = 5–6).

In the presence of phosphatase inhibition (0.1 μM okadaic acid; Lin et al., 2003), bath application of Mg-ATP (0.5 mM) totally rescued the channel rundown (Fig. 6 A, fifth row), which likely reflects channel activation by PIP₂ that is being regenerated via PI4KIIα (Yaradanakul et al., 2007). Moreover, PIP₂ antibodies (monoclonal 1:1,000) applied on top of Mg-ATP to the cytosolic side of the plasma membrane dropped NP₀ to <35% of control, strongly suggesting the involvement of endogenous PIP₂ in controlling BK channel activity in the native membrane. Finally, cotransfection of HEK293 cells with cbv1+β₁ channels and PI4kinaseIIα resulted in robust potentiation of NP₀ (Fig. 6 B). Because transfection of PI4KIIα leads to increased PIP₂ levels (Yaradanakul et al., 2007), the result supports the notion that augmentation in membrane PIP₂ levels leads to increased cbv1+β₁ NP₀.

The possibility of a modulatory role of endogenous PIP₂ on BK channels was also tested by using pharmacological manipulations of macroscopic current in intact,
K⁺ current in intact cerebral artery myocytes. These currents were totally suppressed by 0.3 μM paxilline (Fig. 7E) or 0.1 μM iberiotoxin (not depicted), identifying the PLC-regulated current as of the BK type (Weiger et al., 2002).

Under block of PKC and SR Ca²⁺-ATPase (final targets of IP3 and DAG; Fig. 8), the potentiation of current that results from PLC inhibition could be attributed to (a) buildup of PIP₂ and related phosphoinositide or (b) depletion of IP3 and DAG with loss of a putative direct inhibition of the channel caused by one or both metabolites. IP3, however, potentiates myocyte BK channels (Cai et al., 2005). On the other hand, 2 μM DOG (a cell-permeable DAG analogue) applied to the cytosolic side of I/O patches excised from cerebral artery myocytes evoked no major effect on BK channel activity, with NP of DOG reaching 78.4 ± 2.4% of controls (n = 4). Therefore, the dramatic increase in BK current caused by PLC inhibition in addition to the PI3kinase block has to be primarily attributed to a direct PIP₂ activation of BK channels. Consistent with this interpretation, preincubation with paxilline precluded PI3kinase and/or PLC inhibition from affecting current (Fig. 7, I and J). Collectively, the results in freshly isolated cerebral artery myocytes reinforce the idea that endogenous PIP₂ controls BK currents in the myocyte membrane.

PIP₂ Regulates Cerebrovascular Tone via BK Channels

We next determined any possible contribution of PIP₂ direct modulation of myocyte BK currents to modifications in vascular tone using endothelium-free, pressurized freshly isolated myocytes. We used perforated patches to keep the intracellular milieu intact and recorded total outward currents in PSS containing 0.1 mM niflumic acid to block Ca²⁺-activated Cl⁻ channels (Leduc et al., 2005) and 5 mM 4-aminopyridine to block voltage-gated K⁺ channels other than BK (Thebaud et al., 2004). Under these conditions, myocytes displayed noninactivating outward currents (e.g., 483 pA peak amplitude at 100 mV; Fig. 7, A and F), their major component reported to be the BK current (Catacuzzeno et al., 2000).

Inhibition of PKC (2 μM Ro31-8220; Barman et al., 2004) combined with a block of SR Ca²⁺-ATPase (0.2 μM thapsigargin; Goforth et al., 2002) caused a mild but consistent increase in current (Fig. 7, B and G), suggesting that PKC inhibition of myocyte BK channels (Barman et al., 2004) prevails over channel activation by sarcoplasmic Ca²⁺ (Goforth et al., 2002) (Fig. 8). To build up membrane PIP₂, we inhibited PLC (25 μM U73122; Wilkerson et al., 2006), the major PIP₂-metabolizing enzyme (Tolloczko et al., 2002). This treatment, however, can reroute PIP₂ toward formation of PI3 kinase-mediated IP₃ (Fruman et al., 1998; Suh et al., 2006), a powerful BK channel activator (Fig. 2). Thus, we first blocked PI3kinase (5 nM wortmannin; Arcao and Wymann, 1993), which mildly increased current, likely due to PI₃-P₃ buildup (Fruman et al., 1998; Suh et al., 2006). Subsequent PLC inhibition caused a dramatic increase in both activation slope and amplitude of current (Fig. 7 D), with peak amplitude reaching 6,608.5 ± 1,983.1% of control (n = 4). The result indicates that PLC tonically controls the noninactivating K⁺ current in intact cerebral artery myocytes. These currents were totally suppressed by 0.3 μM paxilline (Fig. 7E) or 0.1 μM iberiotoxin (not depicted), identifying the PLC-regulated current as of the BK type (Weiger et al., 2002).

Under block of PKC and SR Ca²⁺-ATPase (final targets of IP3 and DAG; Fig. 8), the potentiation of current that results from PLC inhibition could be attributed to (a) buildup of PIP₂ and related phosphoinositide or (b) depletion of IP3 and DAG with loss of a putative direct inhibition of the channel caused by one or both metabolites. IP₃, however, potentiates myocyte BK channels (Cai et al., 2005). On the other hand, 2 μM DOG (a cell-permeable DAG analogue) applied to the cytosolic side of I/O patches excised from cerebral artery myocytes evoked no major effect on BK channel activity, with NP of DOG reaching 78.4 ± 2.4% of controls (n = 4). Therefore, the dramatic increase in BK current caused by PLC inhibition in addition to the PI3 kinase block has to be primarily attributed to a direct PIP₂ activation of BK channels. Consistent with this interpretation, preincubation with paxilline precluded PI3 kinase and/or PLC inhibition from affecting current (Fig. 7, I and J). Collectively, the results in freshly isolated cerebral artery myocytes reinforce the idea that endogenous PIP₂ controls BK currents in the myocyte membrane.

PIP₂ Regulates Cerebrovascular Tone via BK Channels

We next determined any possible contribution of PIP₂ direct modulation of myocyte BK currents to modifications in vascular tone using endothelium-free, pressurized
maximal contraction and dilation being obtained by perfusing the vessel with 60 mM KCl at the beginning, and Ca\textsuperscript{2+}-free solution at the end of each experiment, respectively (Fig. 9A). Under block of PKC and SR Ca\textsuperscript{2+}-ATPase, PLC inhibition, which increased BK current (Fig. 7), increased diameter (+15.1 ± 0.1%; n = 3) (Fig. 9A). Subsequent PI3 kinase inhibition caused an additional, mild dilation (+5.2 ± 2.2%; n = 3), consistent with the mild increase in BK current caused by this treatment (Fig. 7). Data strongly suggest that PIP\textsubscript{2} and/or other membrane phosphoinositides directly modulate myogenic tone of cerebral arteries. In the presence of paxilline, a selective BK channel blocker, neither PLC inhibition nor PI3 kinase block caused major dilation: 5.5 ± 4.4% (n = 4) and 6.4 ± 4.3% (Fig. 9B), indicating that the phosphoinositide effect on myogenic tone is primarily mediated via BK channels.

DISCUSSION

Our study identifies a new mechanism by which membrane PIP\textsubscript{2} controls smooth muscle BK currents and, thus, vascular tone: an increase in channel steady-state activity due to an apparent direct interaction between PIP\textsubscript{2} and the BK protein complex. Thus, PIP\textsubscript{2} controls BK currents in cerebral artery myocytes via two mechanisms (Fig. 8): (1) indirect, which involves the well-known PLC signaling pathway, and (2) direct, through amplification of Ca\textsuperscript{2+}-driven gating of the BK channel with consequent increase in P\textsubscript{o}. This amplification is secondary to recognition of negative charge and the inositol moiety in the PIP\textsubscript{2} headgroup by the BK channel/H9251 subunit, with drastic potentiation by the smooth muscle-abundant accessory \(1\alpha H9252\) subunit.

Figure 9. Endogenous PIP\textsubscript{2} dilates cerebral arteries via BK channels. Diameter traces from de-endothelialized arteries after developing myogenic tone. (A) In the presence of 2 μM Ro 31-8220 and 0.2 μM thapsigargin, PLC inhibition (25 μM U73122) causes a robust dilation (+15.1%), which is further increased by 5 nM wortmannin. Maximal dilation is evoked by Ca\textsuperscript{2+}-free solution. (B) BK channel block (0.3 μM paxilline) reduces diameter (−13%) and almost totally blunts U73122 and wortmannin actions. In A and B, a vertical line indicates the time at which Ro 31-8220 and thapsigargin were applied; horizontal lines help to visualize diameter changes. (C) Averaged diameters; **, P < 0.01; *, P < 0.05; n = 4.

Our study identifies a new mechanism by which membrane PIP\textsubscript{2} controls smooth muscle BK currents and, thus, vascular tone: an increase in channel steady-state activity due to an apparent direct interaction between PIP\textsubscript{2} and the BK protein complex. Thus, PIP\textsubscript{2} controls BK currents in cerebral artery myocytes via two mechanisms (Fig. 8): (1) indirect, which involves the well-known PLC signaling pathway, and (2) direct, through amplification of Ca\textsuperscript{2+}-driven gating of the BK channel with consequent increase in P\textsubscript{o}. This amplification is secondary to recognition of negative charge and the inositol moiety in the PIP\textsubscript{2} headgroup by the BK channel α subunit, with drastic potentiation by the smooth muscle-abundant accessory β\textsubscript{1} subunit.

**PIP\textsubscript{2} Direct Mechanism: Molecular Players**

Most biological actions of PIP\textsubscript{2} that occur through binding to specific protein sites require electrostatic interactions between negative charges in the PIP\textsubscript{2} headgroup and positive charges in the target site. This has been demonstrated for PIP\textsubscript{2} direct regulation of several types of ion channels (Fan and Makielski, 1997; Shyng et al., 2000; Suh and Hille, 2005; Rohács, 2007; Voets and Nilius, 2007). The critical dependence of BK channel activation on phosphoinositide’s negative charges demonstrated here supports the hypothesis of a protein recognition site being involved. In addition, data show that PI5P is more effective than 1,2-dipalmitoyl-l-phosphatidyl-d-myo-inositol 4-monophosphate (PI4P) in increasing BK NP\textsubscript{o} (unpublished data). This PIP isomer specificity also implicates a defined protein site in PIP\textsubscript{2} action. While PIP\textsubscript{2} activation of BK is drastically amplified by β\textsubscript{1} (Fig. 3, E and F), it is evident in homomeric cbv1 channels (Fig. 3, B and F). This result indicates that cbv1 is sufficient to respond to PIP\textsubscript{2} and suggests that the subunit contains a PIP\textsubscript{2} binding site(s).
PIP\textsubscript{2} interacts with Kir channels at several intracellular sites, most of which have positively charged residues (Shyng et al., 2000; Suh and Hille, 2005). Similarly, positive residues at the end of S6 are thought to contribute to the PIP\textsubscript{2} sensitivity of KCNQ1/KCNE1 channels (Loussouarn et al., 2003). Positive residues at an equivalent location in TRP channels are also proposed for recognition of PIP\textsubscript{2} (Rohács, 2007). It is noteworthy that Ala substitutions of RKK in the equivalent region of cbv1 drastically decreased PIP\textsubscript{2} action (Fig. 3 D) without modifying basic current phenotype (Fig. 3 C). Thus, the reduced PIP\textsubscript{2} action in the mutant is not due to an overall change in cbv1 protein conformation caused by the mutation. Rather, neutralization of RKK specifically prevents cbv1 from effectively sensing PIP\textsubscript{2}. Collectively, our data suggest that PIP\textsubscript{2} directly interacts with cbv1 at an RKK cluster via electrostatic interactions to activate BK channels. In the absence of crystallographic data of BK channels, it is not possible to determine whether the RKK cluster is an actual PIP\textsubscript{2} binding site or a transducing region that connects the channel gate with a PIP\textsubscript{2} binding site(s) located elsewhere in the cbv1 (α) subunit. From 25 crystallographic structures of proteins that bind PIP\textsubscript{2}, however, several major criteria for a PIP\textsubscript{2} binding site emerge: (1) it must contain at least two positively charged residues (Arg and Lys); (2) among these, at least one should be Arg; (3) the presence of at least one hydrophobic residue nearby; (4) involvement of at least five interacting residues (Rosenhouse-Dantsker and Logothetis, 2007). Remarkably, the RKK and its nearby context in cbv1 (Fig. 3 A) fulfill three out of these four criteria. While we did not test the fourth criterion, we note that the RKKcbv1AAA mutant is not completely insensitive to PIP\textsubscript{2} (Fig. 4 D), suggesting that residues other than the RKK triplet also play a role in PIP\textsubscript{2} sensing. Interestingly, cbv1 contains a large intracellular structure with 110 positive residues, many of which are potentially able to interact with membrane PIP\textsubscript{2}. Future studies using alanine scanning mutagenesis will help to clarify whether one or more of these residues are involved in PIP\textsubscript{2} regulation of BK channel activity.

A variety of BK α subunit isoforms resulting from alternative splicing of Slo1 have been identified in different tissues and species (Xie and McCobb, 1998; Salkoff et al., 2006), and their PIP\textsubscript{2} sensitivity remains to be determined. Following expression in X. oocytes, bslo (from bovine aorta) channel activity is significantly increased by acute exposure to brain phosphoinositides (Liu et al., 2003). Notably, bslo subunits also contain the RKK sequence in their S6–S7 cytosolic loop.

In contrast to Slo1, BK β\textsubscript{1} subunit cytosolic regions do not include RKK sequences. However, BK β\textsubscript{1} cytosolic C and N ends both contain scattered, positively charged residues that are absent in BK β\textsubscript{4} subunits (Behrens et al., 2000; Brenner et al., 2000). Thus, we cannot currently rule out that PIP\textsubscript{2} occupation of additional binding sites located in BK β\textsubscript{1} accessory subunits contributes to amplification of PIP\textsubscript{2} action on cbv1 activity by β\textsubscript{1} subunits. On the other hand, β\textsubscript{1} subunit-induced enhancement of the BK channel’s apparent calcium sensitivity has been primarily attributed to changes in voltage-dependent gating of Slo1 (Bao and Cox, 2005), which occur with reduction in both gating charge and channel intrinsic open-to-closed equilibrium, and enhanced coupling between voltage sensing and channel opening (Orio and Latorre, 2005; Wang and Brenner, 2006). Disregarding the mechanistic underpinnings, amplification of PIP\textsubscript{2} action on cbv1 activity by BK β\textsubscript{1} subunits appears to indicate that conformational changes in cbv1 that occur upon PIP\textsubscript{2} interaction with RKK in the channel S6–S7 cytosolic loop are functionally coupled to the channel voltage sensor movements.

**PIP\textsubscript{2} Direct Activation of BK Channels Differs from that Caused by Other Negatively Charged Lipids**

BK channel steady-state activity is regulated by negatively charged lipids other than PIP\textsubscript{2}, the best studied group being fatty acids (FA). FA direct activation of BK channels, however, differs from PIP\textsubscript{2}-induced activation in several critical aspects. First, FAs increase P\textsubscript{o} without a major effect, if any, on channel mean open time (Clarke et al., 2002). In contrast, PIP\textsubscript{2} drastically enhances mean open time by stabilizing medium and long channel openings (Fig. 5 C). Second, palmitoylcoenzyme-A (that is, “a membrane-impermeable fatty acid”) is effective only when applied to the extracellular membrane leaflet (Clarke et al., 2003). PIP\textsubscript{2}, however, activates BK channels by accessing the channel via its intracellular side (Fig. 1 C), which likely allows phosphoinositide sensing by the RKK cluster of positive residues in the cytosolic S6–S7 linker of the channel (Fig. 3, A–D). Third, FA action appears Ca\textsuperscript{2+}-dependent (Clarke et al., 2002). In contrast, PIP\textsubscript{2} increases activity through amplification of Ca\textsuperscript{2+}-driven gating. This mechanism is supported by the following observations: (a) PIP\textsubscript{2} action in solutions having zero nominal Ca\textsuperscript{2+}, plus 5 mM EGTA to chelate trace amounts of the metal is negligible (Fig. 3 A); (b) PIP\textsubscript{2} action is blunted by shielding negative charge with 300 mM Na\textsuperscript{+} in the bath solution (Vaithianathan, T., P. Liu, and A. Dopico. 2006. Society for Neuroscience. Online. 627.5); (c) PIP\textsubscript{2} causes a parallel shift in the G\textsubscript{max}–voltage plot (Fig. 3 C); and (d) PIP\textsubscript{2} action is potentiated by β\textsubscript{1}, but not β\textsubscript{4}, subunits. Interestingly, PIP\textsubscript{2} readily and consistently activates BK channels only if applied to the cytosolic side of the channel, where the Ca\textsuperscript{2+} sensors are located (Cai et al., 2005).

Finally, BK channel activation by arachidonic acid and analogues has recently been linked to the ability of these FAs to remove β\textsubscript{c} or β\textsubscript{d}-mediated BK inactivation. Thus, these FAs fail to modulate slo1 channel function (Sun et al., 2007). In contrast, we demonstrate that (a) the BK channel-forming subunit is sufficient for PIP\textsubscript{2} action; (b) this action is amplified by β\textsubscript{1} subunits, which do not
introduce channel inactivation (Meera et al., 1996; Brenner et al., 2000), and (c) PIP2 action is poor on native skeletal muscle BK channels (Fig. 4) where β3 subunits are significantly expressed (Behrens et al., 2000).

As found here with PIP2, lithocholate and other structurally related cholane derivatives directly increase BK NP, by modifying both open and closed time distributions (Bukiya et al., 2007). Differing from PIP2 action, however, lithocholate fails to activate cbv1 channels even at concentrations that are maximally effective on native BK or cbv1-β3 channels; the presence of the β3 subunit is required for lithocholate to activate BK channels (Bukiya et al., 2007). In conclusion, PIP2 direct activation of BK channels shows unique structural and functional features when compared with the direct activation of these channels by other negatively charged lipids.

Pathophysiological Implications

Our study demonstrates for the first time that BK channels belong to the group of ion channels that PIP2 directly regulates (Hilgemann and Ball, 1996; Fan and Makielski, 1997; Runnels et al., 2002; Rohács et al., 2003; Chemin et al., 2003; Suh and Hille, 2005; Brauchi et al., 2007; Hilgemann, 2007; Rohács 2007; Voets and Nilius, 2007). An important addition to current knowledge on PIP2 regulation of ion channels is that the final PIP2 effect is critically determined by channel accessory subunits, and such a mechanism can be subunit specific. Moreover, the differential expression of BK accessory subunits across tissues (Behrens et al., 2000; Brenner et al., 2000; Orio et al., 2002) raises the possibility that a direct PIP2 modulation of BK channel function is specifically relevant in tissues where the β3 subunit is highly expressed. Indeed, while PIP2 robustly activates native BK channels in vascular smooth muscle (Fig. 1), where β3 subunits are highly expressed, it mildly activates native BK channels in skeletal muscle, where β1 subunits are barely detected (Behrens et al., 2000).

In conclusion, we demonstrate a new mechanism for modulating BK currents in cerebrovascular smooth muscle: PIP2 direct modulation of BK channel gating. Our data, obtained with recombinant proteins, cells, and intact arteries from the cerebrovascular system, opens the possibility of determining the role of the direct interaction between PIP2 and BK channels in pathophysiological processes leading to disease, such as cerebrovascular spasm and ischemic stroke. Moreover, determining the structural requirements in PIP2 and the BK complex for modulating channel function may pave the way for designing new agents to control myogenic tone.

The authors thank Donald Hilgemann for helpful criticism and David Armbruster for critically reading the manuscript.

This work was supported by grants AA11560 and HL77424 (A. Dopico), GM-61943 and HL-58135 (Z. Fan), and AHA SouthEast Postdoctoral Fellowships (J. Liu, P. Liu).
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