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Do Anionic Phospholipids Serve as Cofactors or Second Messengers for the Regulation of Activity of Cloned ATP-Sensitive K+ Channels?

Kathryn V. Quinn,* Yi Cui,* Jonathan P. Giblin, Lucie H. Clapp, Andrew Tinker

Abstract—The regulation of ion channels by anionic phospholipids is currently very topical. An outstanding issue is whether phosphatidylinositol 4,5-diphosphate and related species act as true second messengers in signaling or behave in a manner analogous to an enzymatic cofactor. This question is especially pertinent regarding ATP-sensitive K+ channels in smooth muscle, for which there is substantial literature supporting inhibitory regulation by hormones. In this study, we have examined regulation of the potential cloned equivalents of the smooth muscle ATP-sensitive K+ channel (SUR2B/Kir6.1 and SUR2B/Kir6.2). We find that both can be inhibited via the $G_{q/11}$-coupled muscarinic M3 receptor but that the pathways by which this occurs are different. Our data show that SUR2B/Kir6.1 is inhibited by protein kinase C and binds anionic phospholipids with high affinity, such that potential physiological fluctuations in their levels do not influence channel activity. In contrast, Kir6.2 is not regulated by protein kinase C but binds anionic phospholipids with low affinity. In this case, phosphatidylinositol 4,5-diphosphate and related species have the potential to act as second messengers in signaling. Thus, Kir6.1 and Kir6.2 are regulated by distinct inhibitory mechanisms. (*Circ Res*. 2003;93: 646-655.)

Key Words: ATP-sensitive K+ channels ■ ion channel regulation ■ anionic phospholipids ■ protein kinase C

Recently, there has been great interest in the role of anionic phospholipids in determining the activity of ion channels and transporters.[1,2] Specifically, it has been proposed that phosphatidylinositol (PtdIns) 4,5-diphosphate [PtdIns(4,5)P$_2$] depletion, such as may occur after the activation of phospholipase C (PLC) through a $G_{q/11}$-coupled receptor, is an important regulatory mechanism involved in ATP-sensitive K+ (K_{ATP}) channel inhibition.[2–5] However, PLC also leads to the production of inositol triphosphate and diacylglycerol, and the latter leads to the activation of protein kinase C (PKC). The major issue is whether depletion of PtdIns(4,5)P$_2$ and related species or activation of PKC is the prominent signaling mechanism. These issues are especially pertinent in smooth muscle, for which there is a substantial body of work showing hormonal regulation of K_{ATP} currents. For example, in native vascular smooth muscle tissues, it is clear that vasoconstrictors, in particular, angiotensin II, inhibit the channel by activating PKC.[6] How then do we reconcile the recent demonstration of the importance of anionic phospholipids in channel regulation and the established literature, with agreement from a number of laboratories, pointing to the importance of PKC signaling in the regulation of native K_{ATP} channels? (see Quayle et al[6] for review) It is this question that we address in the present study, which involves the cloned equivalents of the vascular K_{ATP} channel.

K_{ATP} is an octameric protein complex composed of pore-forming subunits (Kir6.1 and Kir6.2), a member of the inwardly rectifying family of K+ channels (Kir), and the sulfonylurea receptor subunit, a member of the ATP-binding cassette family of proteins (SUR1, SUR2A, and SUR2B). The assembly of a particular pore-forming subunit with a particular SUR generates currents with a characteristic single-channel conductance, nucleotide regulation, and pharmacology.[7–9] It has been proposed that SUR2B/Kir6.1 is the molecular counterpart of the vascular smooth muscle K_{ATP} current, with properties akin to that of the nucleotide diphosphate–sensitive K+ (K_{NDP}) current.[10,11] SUR2B/Kir6.2 has more classic properties and is proposed to constitute the K_{ATP} current in nonvascular smooth muscles.[12] It is not clear yet whether this broad generalization is correct, but studies with Kir6.1- and SUR2-knockout mice and our own work involving a primary pulmonary artery smooth muscle cell line are consistent with the native vascular channel being composed of SUR2B/Kir6.1.[13–15] In the present study, we examine the regulation of the cloned equivalents of the vascular K_{ATP} channel (SUR2B/Kir6.1 and SUR2B/Kir6.2) by anionic phospholipids and PKC.
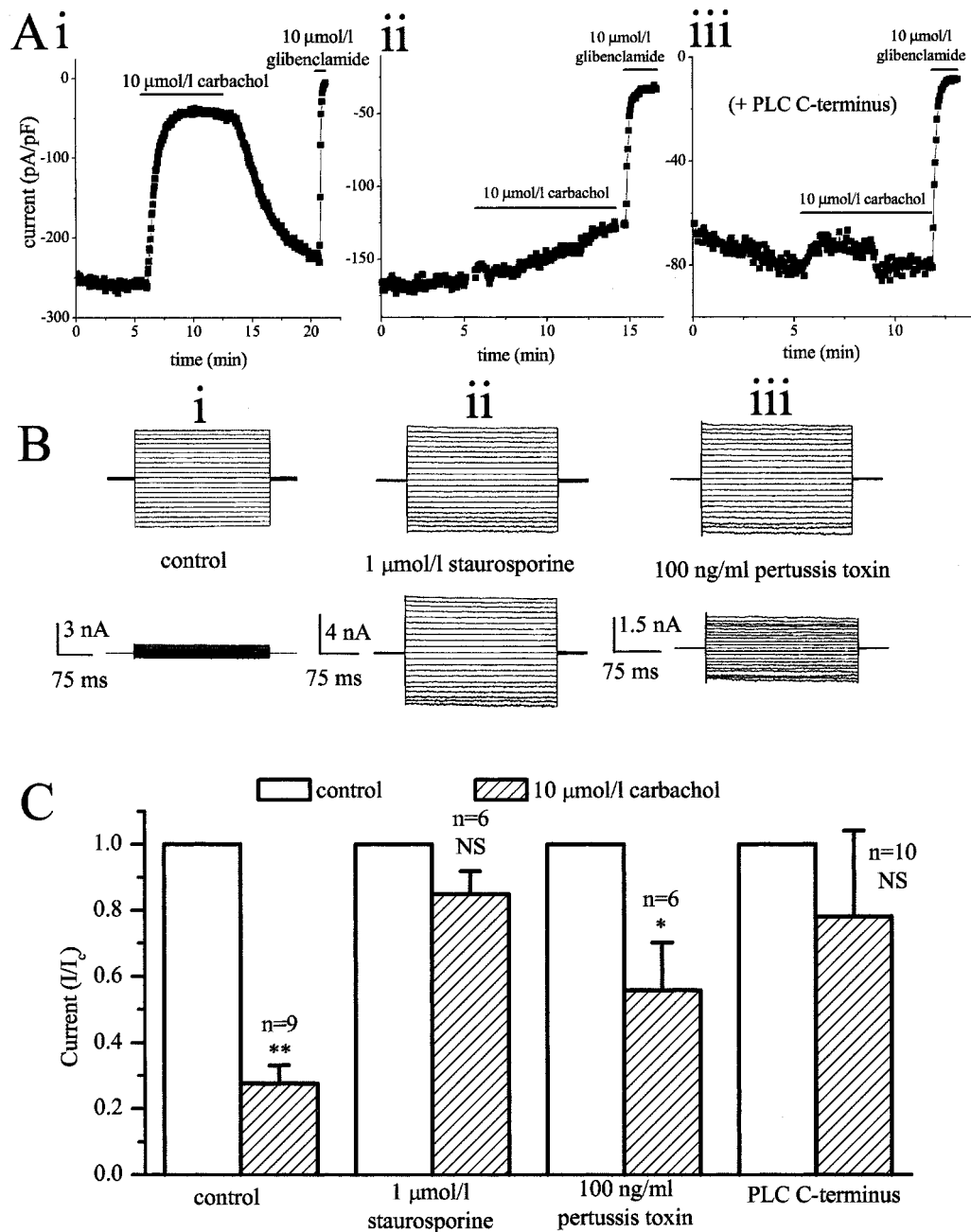
Figure 1. Effects of M3 receptor activation on SUR2B/Kir6.1 currents. HEK293 cells stably expressing SUR2B/Kir6.1 were transiently transfected with M3 receptor cDNA. Whole-cell pipette solutions were supplemented with 1 mmol/L MgATP, 500 μmol/L Na$_2$UDP, and 100 μmol/L Na$_2$GTP. Recordings were made in a symmetrical K$^+$ gradient (140 K$^+$) and began 5 minutes after break-in (t=0). A, Time series of whole-cell currents elicited during 25-ms voltage steps (every 3 seconds) to -60 mV from a holding potential of 0 mV. Recordings shown are from a control cell (i), from a cell pretreated for 15 minutes with 1 μmol/L staurosporine (ii, present throughout and superfused in the bath solution), and from a cell in which the PLCβ C-terminus was overexpressed (iii). B, Representative current-voltage traces are shown from control cells (i), after treatment with 1 μmol/L staurosporine (ii), and after overnight incubation with 100 ng/mL pertussis toxin (iii). Upper traces show control current, and lower traces were recorded in the presence of 10 μmol/L carbachol. C, Mean normalized currents ($I/I_{control}$) recorded in steady-state control conditions (open bars) and after the addition of carbachol (hatched bars) in the absence and presence of 1 μmol/L staurosporine, after overnight treatment with 100 ng/mL pertussis toxin or with overexpression of PLCβ C-terminus (current densities: control -273 ± 43.7 pA/pF, carbachol -75.6 ± 22.8 pA/pF; for staurosporine, control -157 ± 34.8 pA/pF, carbachol -133 ± 26.9 pA/pF; for pertussis toxin, control -110.1 ± 27.5 pA/pF, carbachol -60.6 ± 15.8 pA/pF; and for overexpression of PLCβ C-terminus, control -85.2 ± 9.8 pA/pF, carbachol -67.3 ± 11.23 pA/pF). *$P<0.05$, **$P<0.01$.

Materials and Methods

Molecular Biology

Standard subcloning techniques were used throughout. The M3 receptor and PLCβ C-terminus were used as previously de-scribed.[16] The pleckstrin homology (PH) domain from PLCδ (PH-CFP construct) was kindly provided by Dr Tobias Meyer (Stanford University Medical School, Stanford, Calif). Polymerase chain reaction fragments corresponding to the C-termini of rat Kir6.1 (amino acids 179 to 424) and mouse Kir6.2 (amino acids
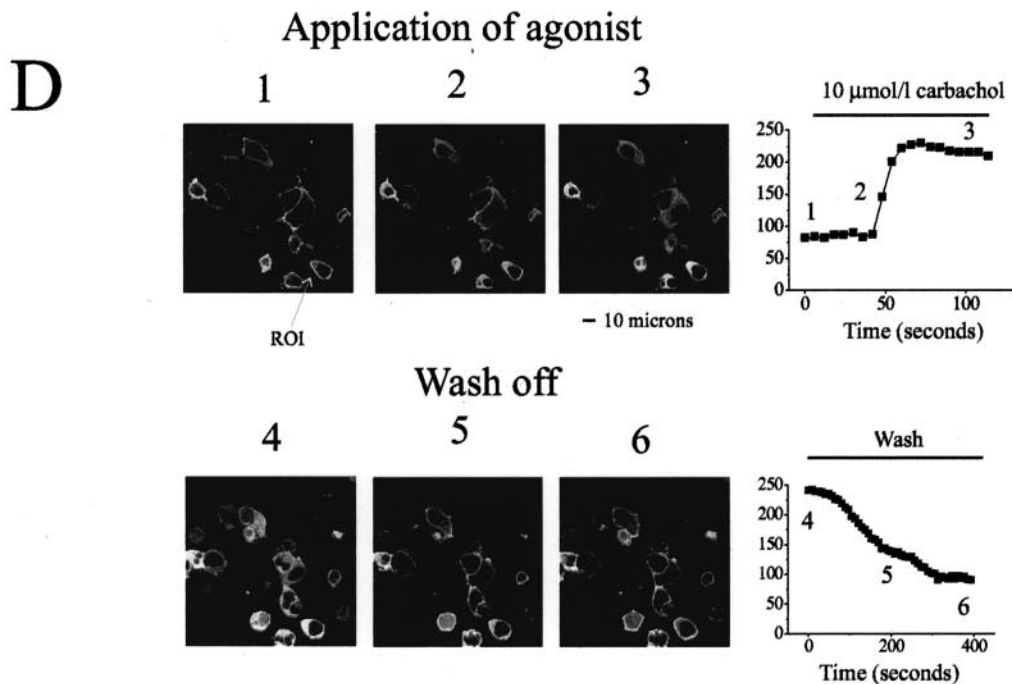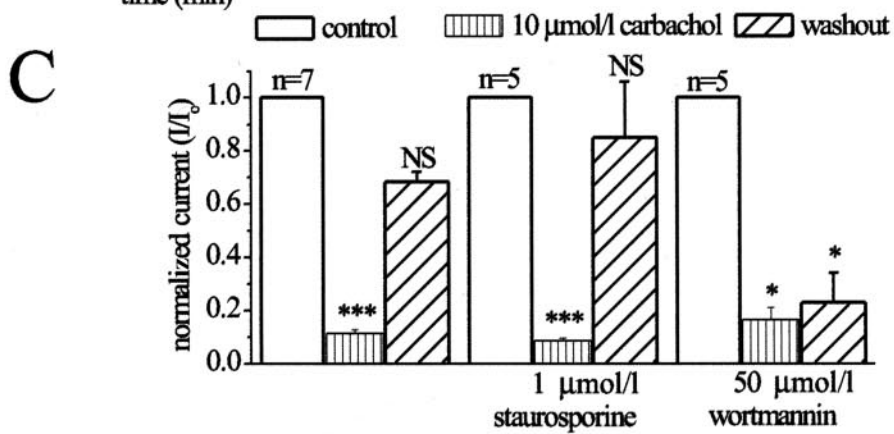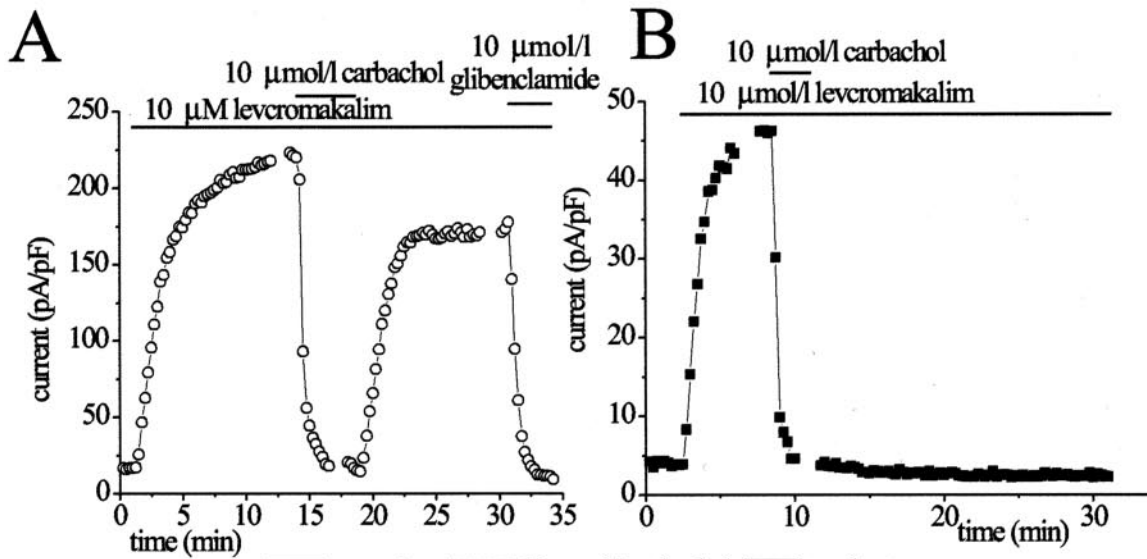
189 to 391) were subcloned into the pMal-c2x vector (New England Biolabs).

Production and Purification of MBP Fusion Proteins

Fusion protein vectors were transformed into BL21(DE3) *Escherichia coli*. Fusion protein expression was induced according to the manufacturers' instructions, except induction occurred at 20°C overnight. Cells were harvested by centrifugation, and the cell pellet was washed with PBS/5 mmol/L EDTA (column buffer) and resuspended in column buffer containing protease inhibitors (Roche complete protease inhibitor cocktail, EDTA free) before freezing. The cell suspension was rapidly thawed at 37°C and sonicated on ice, and insoluble material was removed by centrifugation at 10 000*g* for 30 minutes at 4°C. The supernatant was applied to a column containing a 2-mL bed volume amylose-binding resin and washed with 12 column volumes before elution with column buffer containing 10 mmol/L maltose. Protein-containing fractions were pooled and dialyzed against the column buffer. The protein concentration of the purified fusion proteins was measured, and purity was assessed by SDS-PAGE, followed by Coomassie blue staining. After purification, the C-terminus of Kir6.2 (maltose-binding protein [MBP]-Kir6.2C) was judged to be ≈90% pure, and the C-terminus of Kir6.1 (MBP-Kir6.1C) was judged to be ≈80% pure, with a lower molecular weight species consistently present.

Lipid-Binding Experiments

Nitrocellulose strips spotted with lipids (PIP strips, Molecular Probes Inc) were blocked for 1 hour in a blocking solution composed of 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L KCl, pH 7.4, and 0.1% (vol/vol) Tween containing 3% (wt/vol) BSA with low fatty acid content (<0.02%) at room temperature. After the blocking, the strips were incubated overnight at 4°C with a 0.5 μg/mL solution of the appropriate MBP fusion protein in blocking solution. Strips were then processed as for Western blotting using a rabbit primary antibody to the MBP fusion protein. Strips of nitrocellulose were spotted with L-α-PtdIns(4,5)P$_2$ in a total volume of 2 μL for each spot from a 500 μmol/L stock in chloroform/methanol/H$_2$O (50:50:15).

Phosphorylation Assay

The fusion proteins bound to the amylose resin were washed with 1 mL of 5× HEPES buffer (50 mmol/L HEPES, pH 7.4, 1 mmol/L EDTA, 10% glycerol, 5 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, 1 mmol/L dithiothreitol, and protease inhibitors). Added to 10 μg protein was 20 ng catalytically active PKC (obtained from Calbiochem), 6 μL of 5× buffer (50 mmol/L HEPES,

Figure 2. Effects of M3 receptor activation on SUR2B/Kir6.2 currents. HEK293 cells stably expressing SUR2B/Kir6.2 were transiently transfected with the M3 receptor. Recordings were made in a physiological K$^+$ gradient (see Materials and Methods). A, Time series of current recorded in perforated patch, measured at −5 mV during a 2-second voltage ramp from −100 to 0 mV from a holding potential of −60 mV, every 15 seconds. Levcromakalim was applied in the bath to activate SUR2B/Kir6.2 current before application of carbachol. B, Experiment similar to that shown in panel A, except 50 μmol/L wortmannin was applied 10 minutes before recording began and during the entire experiment. C, I/I$_{control}$ recorded without staurosporine (i; current densities were as follows: control 123±30.7 pA/pF, +carbachol 13.6±3.6 pA/pF, and wash 85.7±23 pA/pF), with staurosporine (ii; current densities were as follows: control 106.9±13.3 pA/pF, +carbachol 8.5±.5 pA/pF, and wash 86.6±17.4 pA/pF), and with 50 μmol/L wortmannin (iii; current densities were as follows: control −23.6±6.4 pA/pF, +carbachol 3.1±0.6 pA/pF, and wash 3.5±1.2 pA/pF). *P<0.05 and ***P<0.001. D, HEK293 cells transfected with PH-CFP and the M3 receptor. Drug was applied at the third time point. This was quantified in one of the cells in the field by measuring the change in fluorescence in a region of interest (ROI) in the cytoplasm.
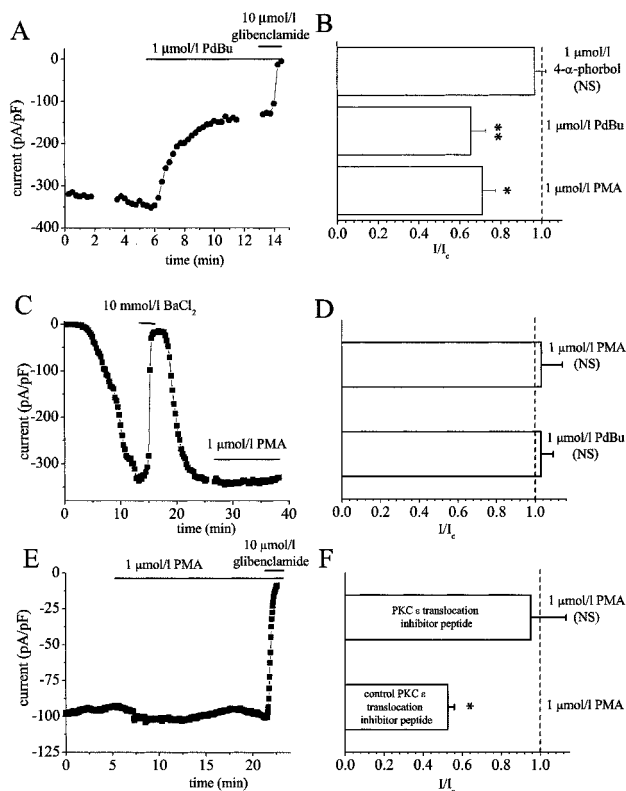
Figure 3. Effects of direct activation of PKC via phorbol esters. Whole-cell pipette solutions were supplemented with 1 mmol/L MgATP and 500 μmol/L Na$_2$UDP. Recordings were begun 5 minutes after break-in. A, Example of whole-cell current recorded from HEK293 cells with stably transfected SUR2B/Kir6.1. Voltage ramps from −100 to 100 mV were applied every 15 seconds from a holding potential of 0 mV, and current at −60 mV during application of 1 μmol/L PdBu is shown. B, Normalized currents recorded at −60 mV after 10-minute application of PMA (control −80.8±15.8 pA/pF, PMA −46.3±7.6 pA/pF; n=13), PdBu (control −128±42.8 pA/pF, PdBu −74±20.2 pA/pF; n=13), and 4α-phorbol (control −199±31.3 pA/pF, 4α-phorbol −188±17.7 pA/pF; n=4). *P<0.05, **P<0.01. C, Example of current recorded from HEK293 stably transfected SUR2B/Kir6.2 during application of PMA. D, I/I$_{control}$ at −60 mV after 10-minute application of PMA (control −608±158 pA/pF, PMA −621±173 pA/pF; n=7) or PdBu (control −763±173 pA/pF, PdBu −739±162 pA/pF; n=8). E, Example of current recorded from HEK293 stably transfected SUR2B/Kir6.1, with 150 μg/mL PKCε translocation inhibitor peptide in the patch pipette. Voltage steps lasting 25 ms from 0 to −60 mV were applied every 3 seconds, and current at −60 mV during application of PMA is shown. F, I/I$_{control}$ at −60 mV after 10-minute application of PMA with either scrambled (control) 150 μg/mL PKCε translocation inhibitor peptide (control −116.4±25.0 pA/pF, PMA −60.2±11.1 pA/pF; n=4) or 150 μg/mL PKCε translocation inhibitor peptide (control −65.5±13.2 pA/pF, PMA −55.3±10.0 pA/pF; n=6). *P<0.05.

1 mmol/L ATP, and 50 mmol/L MgCl$_2$), 10 μL HEPES buffer, 13 μL of 50 mmol/L HEPES, 1.5% Triton, and 1 μL ^{32}P-γ-ATP (omitted in the case of lipid-binding experiments). The sample was incubated at 37°C for 2 hours and then washed five times with 1 mL HEPES buffer. The protein was eluted, run on an SDS-PAGE gel, dried, and subjected to autoradiography. Gel quantification was performed by using Scion image (Scion Corp).

Cell Culture and Transfection

The culture and transfection of HEK293 cells and the use of stable cell lines expressing SUR2B/Kir6.1 and SUR2B/Kir6.2 were as previously described.[17]

Confocal Microscopy

HEK293 cells were imaged at room temperature (20°C) using a Bio-Rad Radiance 2000 laser scanning confocal microscope. Samples were excited using a 458-nm laser line, and images were obtained by using a 500-nm-long band-pass filter. Images were processed and quantified using LaserPix software (Bio-Rad). A commercially available Krebs-Ringer buffer with glucose (K4002, Sigma) was used for perfusion; this was supplemented with 2.6 mmol/L Ca^{2+} and used according to the manufacturer's instructions.

Electrophysiology

Whole-cell and inside-out patch-clamp recordings were performed as previously described.[17,18] Capacitance transients and series resistance in whole-cell recordings were compensated electronically by using amplifier circuitry. Pipette (whole-cell) and bath (inside-out) solutions contained (mmol/L) KCl 107, $MgCl_2$ 1.2, $CaCl_2$ 1, EGTA 10, and HEPES 5 (KOH to pH 7.2, ≈140 mmol/L K^+). In whole-cell studies, bath solutions contained (mmol/L) KCl 140, $CaCl_2$ 2.6, $MgCl_2$ 1.2, and HEPES 5 (pH 7.4); in perforated-patch experiments, solutions contained (mmol/L) NaCl 140, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 2.6, HEPES 5, and D-glucose 8 (pH 7.4). Nucleotide supplementation of the pipette/bath solution is indicated in the legends. The pH of the solution was checked after nucleotide supplementation and adjusted if needed. Whole-cell experiments were dialyzed for at least 5 minutes before the recordings. For perforated-patch experiments, a stock solution of 100 mg/mL amphotericin B in dimethyl sulfoxide was prepared and diluted in a standard pipette solution to give a final concentration of 200 μg/mL amphotericin B. A stable access resistance was obtained after 10 to 15 minutes (≈10 MΩ). Generally, agents were applied to the bath by using a gravity-driven system or were applied locally to a cell via a plastic pipette (MSC-200, IntraCel).

Materials

PtdIns(4,5)P_2 was obtained from Calbiochem, and fresh solutions were made each day. Liposomes were prepared by sonication of the bath solution for 30 minutes. In the electrophysiological experiments, we used the natural derivative with the arachidonyl and stearyl side chains. For the binding assays, we used the synthetic dipalmitoyl-C_{16} derivative. It is worth noting that the commercially available strips used in Figure 7A contain these derivatives. The PtdIns(4,5)P_2 antibody was obtained from Assay Design Inc. The translocation-inhibiting peptide for PKCϵ and the control were obtained from Calbiochem.

Statistical Analysis

Statistical analysis was carried out by using one-way ANOVA or a paired Student t test as appropriate. Statistical significance is indicated in the figures ($P<0.05$, $P<0.01$, and $P<0.001$). Data are presented as mean±SEM. Current density and open probability (NP$_o$) are presented normalized to control, where appropriate, to enable the assessment of the relative magnitude of the responses. The mean raw measurements are given in the text of the figure legends, and the statistical analyses shown were performed on such data.

Results

We used the M3 muscarinic receptor as an example of a $G_{q/11}$-coupled receptor and examined the modulation of SUR2B/Kir6.1 and SUR2B/Kir6.2 stably expressed in HEK293 cells. We have previously shown low levels of endogenous K^+ currents in these cells.[17] The whole-cell configuration of the patch-clamp technique was used to study membrane currents of SUR2B/Kir6.1 after transient expression of the receptor. In Figure 1, the application of carbachol leads to an inhibition of SUR2B/Kir6.1 currents, and this effect was substantially attenuated if cells were incubated with the protein kinase inhibitor staurosporine. The use of

more PKC-selective compounds, such as GF109203X and related agents, led to direct inhibition of channel currents, probably as a consequence of channel block (not shown). Staurosporine did not cause such effects. Overexpression of the PLCβ C-terminus (a GTPase-activating protein for $G_{q/11}$) abolished inhibition, implicating the G protein $G_{q/11}$ (Figure 1). In contrast, pertussis toxin did not significantly modulate the inhibition (Figure 1). The application of carbachol to SUR2B/Kir6.2 transiently expressing M3 led to a variable inhibition when studied with the whole-cell patch configuration. However, using perforated-patch recordings gave robust and pronounced inhibition via the M3 receptor of currents activated by levcromakalim (Figure 2A). In contrast to SUR2B/Kir6.1 currents, inhibition was not sensitive to staurosporine. The application of wortmannin, at concentrations
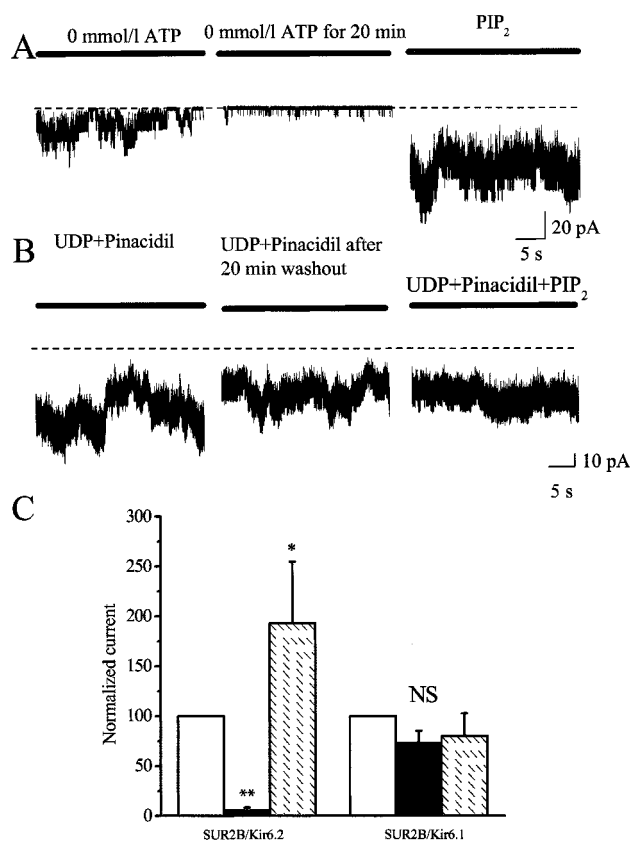
Figure 4. Effects of PtdIns(4,5)P_2 (PIP$_2$) after rundown in excised inside-out patches. A, SUR2B current was fully activated when excised into an ATP-free solution. The current was decreased by >90% after subsequent 20-minute perfusion with the ATP-free solution. However, these channels were reactivated by exposure of the membrane patch to 10 μmol/L PIP$_2$. B, SUR2B/Kir6.1 current activated by 3 mmol/L UDP and 100 μmol/L pinacidil did not show significant rundown after 20-minute perfusion with ATP-free solution. Addition of 10 μmol/L PIP$_2$ had no significant effects on SUR2B/Kir6.1 current activated by UDP+pinacidil. C, Data are shown from a number of such experiments performed as described above. The current amplitude at -60 mV was normalized to that (open bar, as control) measured 1 minute after the membrane patch was excised into ATP-free solution (SUR2B/Kir6.2, n=9) or UDP+pinacidil solution (SUR2B/Kir6.1, n=6). Filled bar indicates 20-minute perfusion with ATP-free solution; hatched bar, addition of PIP$_2$. *$P<0.05$, **$P<0.01$.
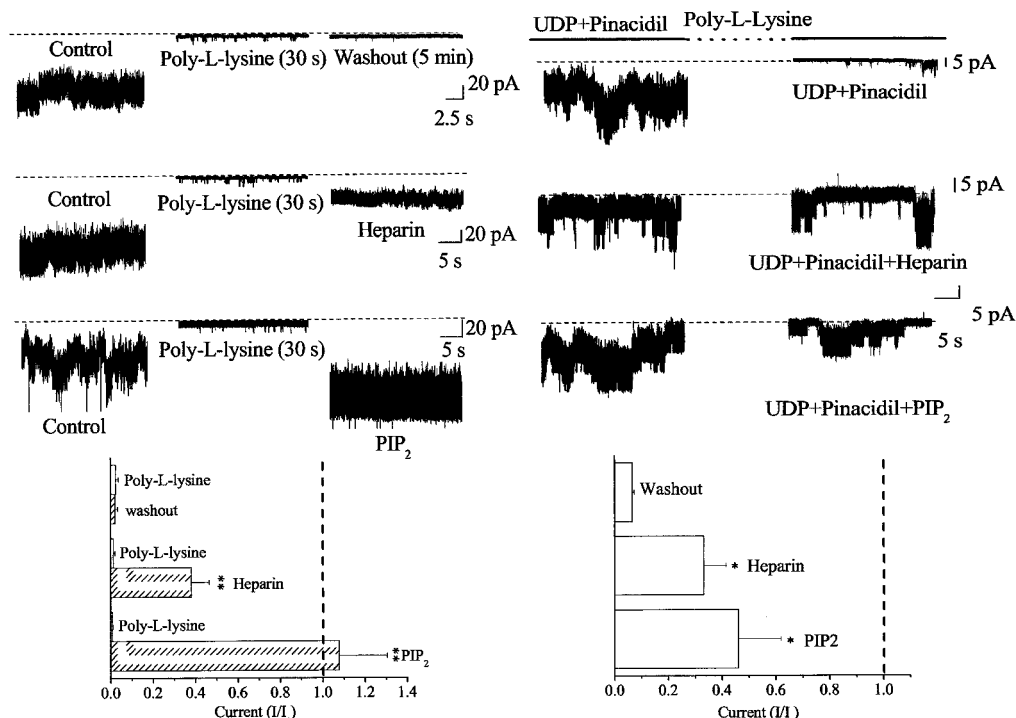
Figure 5. Effects of poly-L-lysine. Currents were recorded in an inside-out patch membrane at −60 mV. Left, Brief (30-second) application of poly-L-lysine (100 μg/mL) caused an instant decrease in Kir6.2/SUR2B current, which was not reversible on washout for 5 minutes (top traces). However, inhibition of currents by poly-L-lysine was reversed partially by heparin at 100 μg/mL (middle traces). Application of PIP$_2$ at 10 μmol/L fully reversed the inhibitory effects of poly-L-lysine (bottom traces) (control, n=9; heparin, n=9; and PIP$_2$, n=10). **$P<0.01$. Right, Effects of poly-L-lysine on Kir6.1/SUR2B currents activated by 3 mmol/L UDP and 100 μmol/L pinacidil. After brief (30-second) treatment with poly-L-lysine at 100 μg/mL, activation of the current by UDP+pinacidil was abolished (top traces). Application of heparin at 100 μg/mL (middle traces) or PIP$_2$ at 10 mmol/L (bottom traces) reversed the inhibitory effects of poly-L-lysine (control, n=8; heparin, n=7; and PIP$_2$, n=9). *$P<0.05$.

(50 μmol/L) that broadly inhibit PtdIns kinases and would thus affect PtdIns(4,5)P$_2$ synthesis, led to irreversible inhibition of SUR2B/Kir6.2 currents after activation of M3 (Figure 2B). In addition, the current density in the presence of the opener was significantly reduced (see Figure 2 legend). Similar experiments with SUR2B/Kir6.1 led to reversible inhibition (not shown). We also confirmed in intact living cells that activation of these pathways via M3 led to PtdIns(4,5)P$_2$ depletion and/or inositol 1,4,5-triphosphate generation by examining the translocation of PH-CFP using confocal microscopy.[19,20] Agonist application led to a reversible translocation of this fluorescent reporter to the cytoplasm (Figure 2C). Thus, SUR2B/Kir6.1 currents can be inhibited by a PKC-dependent mechanism, whereas SUR2B/Kir6.2 currents can be inhibited through G$_{q/11}$-coupled receptors but in a fashion that is independent of protein kinases and dependent on PtdIns lipid synthesis.

We next sought to confirm this by activating PKC in a receptor-independent fashion using phorbol esters. Membrane currents were studied in the whole-cell configuration, and Figure 3 shows that phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate (PdBu) were able to inhibit SUR2B/Kir6.1 currents, whereas they were ineffective when applied to a stable line expressing SUR2B/Kir6.2. The inactive phorbol ester 4α-phorbol didecanoate was ineffective at modulating currents when it was applied to the cell line expressing SUR2B/Kir6.1 (Figure 3). Our pipette solution

has a calculated free Ca^{2+} concentration of 20 nmol/L, implicating a Ca^{2+}-independent isoform of PKC. Thus, we used a PKCϵ translocation inhibition peptide, and this abrogated the inhibition (Figure 3). In addition, phorbol esters were ineffective at modulating Kir6.2 currents when membrane currents were studied with the perforated-patch technique (not shown).

We next examined the role of the substrates of PLC and investigated the potential differences in regulation by anionic phospholipids in inside-out patches expressing SUR2B/Kir6.2 and SUR2B/Kir6.1. One of the indirect hallmarks of such regulation is channel rundown in ATP-free solutions. Figure 4 shows that SUR2B/Kir6.2 channel activity runs down in ATP-free solutions and that this activity is restored by the application of 10 μmol/L PtdIns(4,5)P$_2$. In contrast, SUR2B/Kir6.1-containing complexes do not run down when they are examined under comparable conditions, and the application of PtdIns(4,5)P$_2$ does not increase single-channel NP$_o$. SUR2B/Kir6.2 currents ran down in an equivalent fashion in the presence of UDP and pinacidil (mean NP$_o$ at the start was 0.3337±0.0837, which significantly runs down to 0.0275±0.0269 after 20 minutes; n=6). There are two potential explanations for the inability to demonstrate rundown and the effects of PtdIns(4,5)P$_2$ on SUR2B/Kir6.1 activity. One is that the activity of the channel complex is independent of anionic phospholipids. Alternatively, Kir6.1 channel activity is dependent on anionic phospholipids, but

the interaction is stronger, and the affinity is much higher than that for Kir6.2. We designed a further set of experiments to test this. Poly-L-lysine is able to antagonize the actions of PtdIns(4,5)P$_2$, and heparin is able to reverse this effect. In inside-out patches, poly-L-lysine was able to inhibit both SUR2B/Kir6.2 and SUR2B/Kir6.1. Channel activity could be recovered by the subsequent application of heparin and PtdIns(4,5)P$_2$ (Figure 5). Finally, we examined the consequences of including an anti-PtdIns(4,5)P$_2$ antibody in the recording pipette in the whole-cell configuration. It is thought that the antibody binds to the negatively charged phosphate groups, shielding interaction with basic residues on the channel protein. Dialysis with a pipette solution containing 1 mmol/L ATP led initially to an increase in current, which peaked within 5 minutes and then relaxed to a steady state at ≈10 minutes (this relaxation was more pronounced after inclusion of the antibody). We measured both these parameters in control conditions and after the inclusion of the antibody in the pipette solution. In the latter condition, the antibody significantly reduced the current density in the stable cell line expressing SUR2B/Kir6.2 (Figure 6). In contrast, inclusion of the PtdIns(4,5)P$_2$ antibody in the pipette solution and dialysis for 5 minutes in whole-cell recordings from SUR2B/Kir6.1 did not change the magnitude of the pinacidil-stimulated currents (Figure 6).

A potential explanation for the observed functional phenomena is that the affinity of Kir6.1 for anionic phospholipids is much higher than that for Kir6.2. Thus, we sought more direct biochemical evidence for this. A number of studies have revealed that the major determinants for anionic phospholipid binding are located in the C-terminus of the channel subunit.[2,21–23] Thus, we generated and purified soluble bacterial fusion proteins of these domains linked to the MBP and also the control protein MBP. We used these in overlay binding assays against membranes spotted with a series of anionic phospholipids (100 pmol; see Materials and Methods). We found that MBP-Kir6.1C, but not MBP-Kir6.2C or MBP, bound significantly to these membranes (Figures 7A and 7B). Intriguingly, PtdIns 4-monophosphate [PtdIns(4)P], PtdIns 5-monophosphate [PtdIns(5)P], and PtdIns 3,4-diphosphate [PtdIns(3,4)P$_2$] showed particularly strong (and statistically significant) binding, but PtdIns(4,5)P$_2$ did not. There was also a trend for a number of others to bind above background levels to MBP-Kir6.1C, but this was not statistically significant. Figure 7C shows that it was possible to detect weak but statistically significant binding of MBP-Kir6.1, but not MBP-Kir6.2 or MBP, to nitrocellulose membranes spotted with PtdIns(4,5)P$_2$ at higher concentrations.

Finally, we examined whether the C-terminus of Kir6.1 was a substrate for phosphorylation by PKC and whether this modulated the sensitivity to anionic phospholipids. We performed in vitro phosphorylation assays that showed that MBP-Kir6.1C, but not MBP, was phosphorylated by PKC (Figure 8A). However, phosphorylated protein was still able to bind PtdIns(4)P and PtdIns(5)P at a level equivalent to that for nonphosphorylated protein (Figure 8B).

Discussion

In smooth muscle, there is substantial literature supporting the importance of hormonal regulation of K$_{ATP}$ mediated by
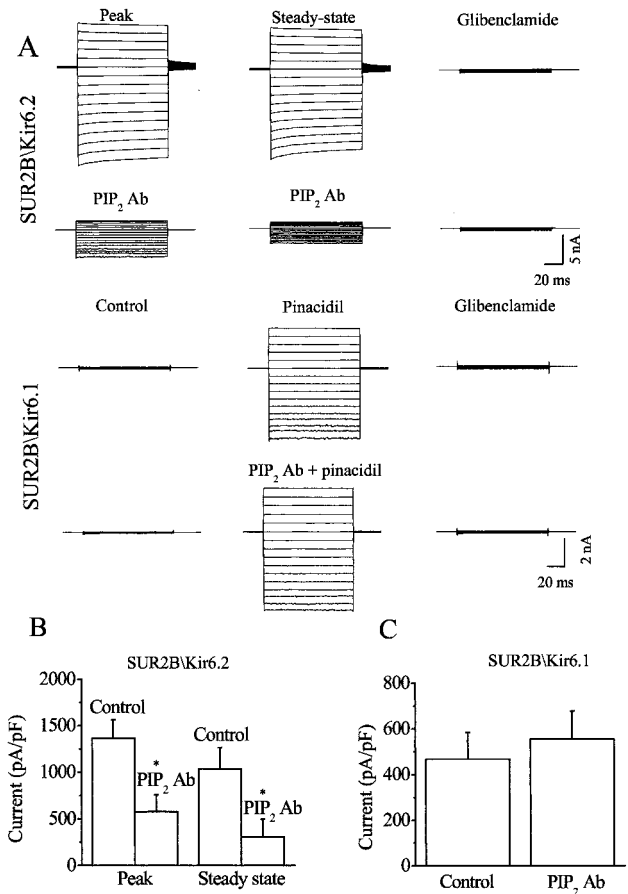

Figure 6. Effects of PIP$_2$ antibody (PIP$_2$ Ab). A, Whole-cell currents were activated by 100-ms voltage steps (range −100 to 50 mV) in 10-mV increments from a holding potential of 0 mV. Dialysis of the cell expressing SUR2B/Kir6.2 with 1 mmol/L ATP pipette solution caused a time-dependent increase in currents inhibited by 10 μmol/L glibenclamide. Inclusion of PIP$_2$ Ab (1:100 dilution) in the pipette solution caused a decrease in current. SUR2B/Kir6.1 currents were activated by perfusion of 100 μmol/L pinacidil in the bath solution, and the pipette solution included 3 mmol/L UDP. Inclusion of the PIP$_2$ Ab (1:100 dilution) in the pipette solution did not affect currents activated by pinacidil. B, Bar chart shows summarized data. Currents were measured at −100 mV and normalized to cell capacitance. Data from peak and steady-state currents are shown. *$P<0.05$. C, Bar chart shows summarized data (n=12).

protein kinases.[6] In contrast, K$_{ATP}$ currents, for example, in cardiac and pancreatic tissues, are more prominently sensitive to the metabolic status of the cell, with conflicting evidence for the role of hormonal regulation and protein kinases (see below). The present study, our own abstracted studies,[24] and studies from other laboratories have indicated that Kir6.1 may be the isoform that is regulated prominently by protein kinases and is responsible for the K$_{ATP/NDP}$ current in smooth muscle.[10,13,14,25] It seems likely that the more metabolically sensitive channels contain the Kir6.2 subunit.[7–9] This is a generalization, and it is apparent that there is overlap. For example, in ventricular cardiomyocytes, PKC has been shown to activate K$_{ATP}$ channels.[26,27] In addition, Kir6.2 may be present in some smooth muscles (eg, the portal vein),[28] and K$_{ATP}$ is metabolically sensitive in some smooth muscles.[6]
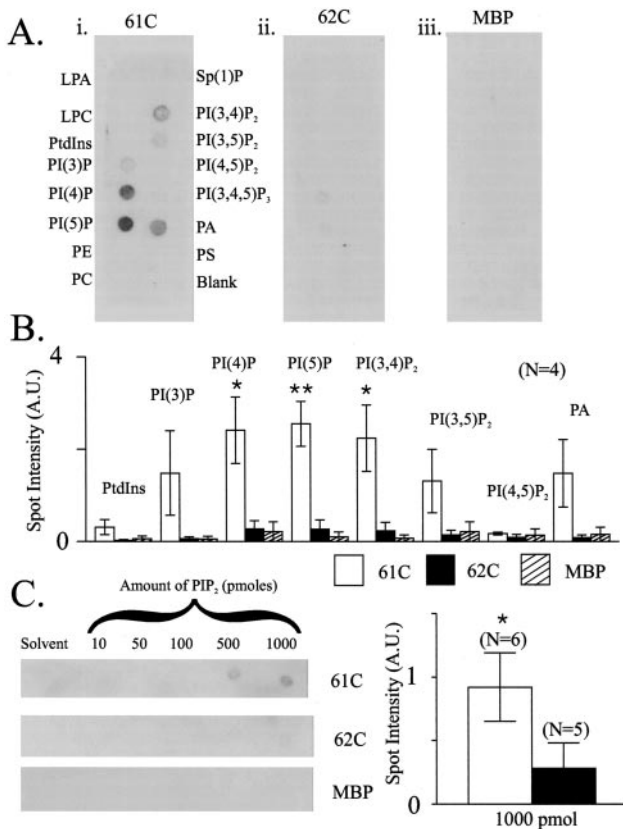
Figure 7. Lipid-binding experiments with Kir6.0 C-terminal MBP fusion proteins. Panel A shows PIP strips probed with anti-MBP antiserum after incubation with 0.5 μg/mL MBP-Kir61C (61C, i), MBP-Kir62C (62C, ii), and MBP (iii). Identical exposures are shown. The intensity of each lipid spot was measured by using Scion image and corrected for background (see Materials and Methods). Each spot equals 100 pmol. LPA indicates lysophosphatidic acid; LPC, lysophosphatidylcholine; PI(3)P, PtdIns(3)P; PI(4)P, PtdIns(4)P; PI(5)P, PtdIns(5)P; PI(3,4)P$_2$, PtdIns(3,4)P$_2$; PI(3,5)P$_2$, PtdIns(3,5)P$_2$; PI(4,5)P$_2$, PtdIns(4,5)P$_2$; PI(3,4,5)P$_3$, PtdIns(3,4,5)P$_3$; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Sp(1)P, sphingosine 1-phosphate; PA, phosphatidic acid; and PS, phosphatidylserine. B, Quantitative analysis is shown for a selection of lipids (n=4). C, Shown are representative dot blots spotted with different amounts of PI(4,5)P$_2$ and a quantitative comparison between the intensities of the 1000-pmol spot in blots probed with MBP-Kir61C and MBP-Kir62C (n=5 or 6). The difference between MBP-Kir62C and MBP was not significant (not shown). One-way ANOVA with Bonferroni correction was used. *$P<0.05$ and **$P<0.01$ compared with MBP and MBP-Kir62C.

The central question that we have attempted to address in the present study is whether the substrate or downstream products of PLCβ are important for signaling to K$_{ATP}$ channels. We have studied the likely cloned equivalents of the smooth muscle channels, namely, SUR2B/Kir6.1 and SUR2B/Kir6.2, because of the background of prominent hormonal regulation in native tissues. Our data reveal novel differences between Kir6.1 and Kir6.2 and show how phospholipid affinity integrates with protein kinase regulation for this important channel family. SUR2B/Kir6.1 is regulated by PKC, and depletion of PtdIns(4,5)P$_2$ or related anionic phospholipids does not have a major role in receptor-mediated regulation. However, we show that the provision of some

PtdIns(4,5)P$_2$ or related anionic phospholipids is necessary for SUR2B/Kir6.1 channel activity. In other words, anionic phospholipids are playing a role that is analogous to an enzymatic cofactor. In addition, we have implicated PKCε as the major PKC isoform responsible for regulation, and this is consistent with studies in vascular smooth muscle.[29] It is also interesting to note that the magnitude of inhibition is greater via M3 than with phorbol esters, suggesting that another pathway, such as PKA, may be involved.[29]

In contrast, SUR2B/Kir6.2 is regulated by PtdIns(4,5)P$_2$ but not by PKC. The regulation of Kir6.2-containing channel complexes seems especially controversial. Our data and those of others show a PKC-independent inhibition of cloned K$_{ATP}$ channel activity.[3–5] This has recently been extended to inhibition via the α$_1$-adrenergic receptor in native ventricular myocytes.[30] In contrast, other investigators have demonstrated a PKC-dependent activation of the native cardiac K$_{ATP}$ channel and its cloned equivalents, SUR2A/Kir6.2 and SUR2B/Kir6.2.[31,32] Such pathways are potentially important, inasmuch as the sarcolemmal K$_{ATP}$ channel is once again assuming importance in cellular protective phenomena.[26,33] The picture is further complicated by the failure to observe any changes in PtdIns(4,5)P$_2$ concentration using biochemical methods in cardiac atrial and ventricular cells after G$_{q/11}$ receptor stimulation.[34]

Emerging from a number of laboratories is a consensus that anionic phospholipids are an important factor necessary for the activity of Kirs and other transporters and channels.[1] In the case of Kirs, it seems there is a direct interaction between the phospholipid and basic residues present on the C-terminus of the channel[1,21] and that this potentially can be regulated by protein kinases.[35] A key point is whether under physiological conditions in native tissues, receptor activation leads to sufficiently large changes in PtdIns(4,5)P$_2$ concentration relative to the EC$_{50}$ for channel activation such that activity is modulated. It is apparent for the inwardly rectifying family of K$^+$ channels that the affinity and kinetics of this interaction can vary. For example, Kir2.1 interacts avidly with PtdIns(4,5)P$_2$, whereas the interaction with Kir3.1/3.4 and Kir2.3 is weaker.[36] Thus, we propose that the affinity for PtdIns(4,5)P$_2$ or related anionic phospholipids is weaker for Kir6.2 than for Kir6.1 and that the EC$_{50}$ for channel activation is numerically higher and within the range for physiological modulation. In contrast, Kir6.1 has much higher affinity, and the variations in PtdIns(4,5)P$_2$ or related anionic phospholipids occur at concentrations that result in saturating activity and thus do not result in changes in activity on receptor activation, even with receptor overexpression. Channel behavior is modulated by phosphorylation by PKC and protein kinase A, and it is also evident that PKC does not seem to act by modulating the anionic phospholipid sensitivity, as has been recently seen with other inward rectifiers.[35] Furthermore, there is evidence of a direct competition between nucleotides and anionic phospholipids over a short C-terminal domain in K$_{ATP}$ channels.[23,37] Our data would explain why Kir6.1-containing channel complexes are less sensitive to inhibition by ATP and thus show basal activity in smooth muscle.[10] In addition, there are some interesting, more speculative features with these data. It is clear that the
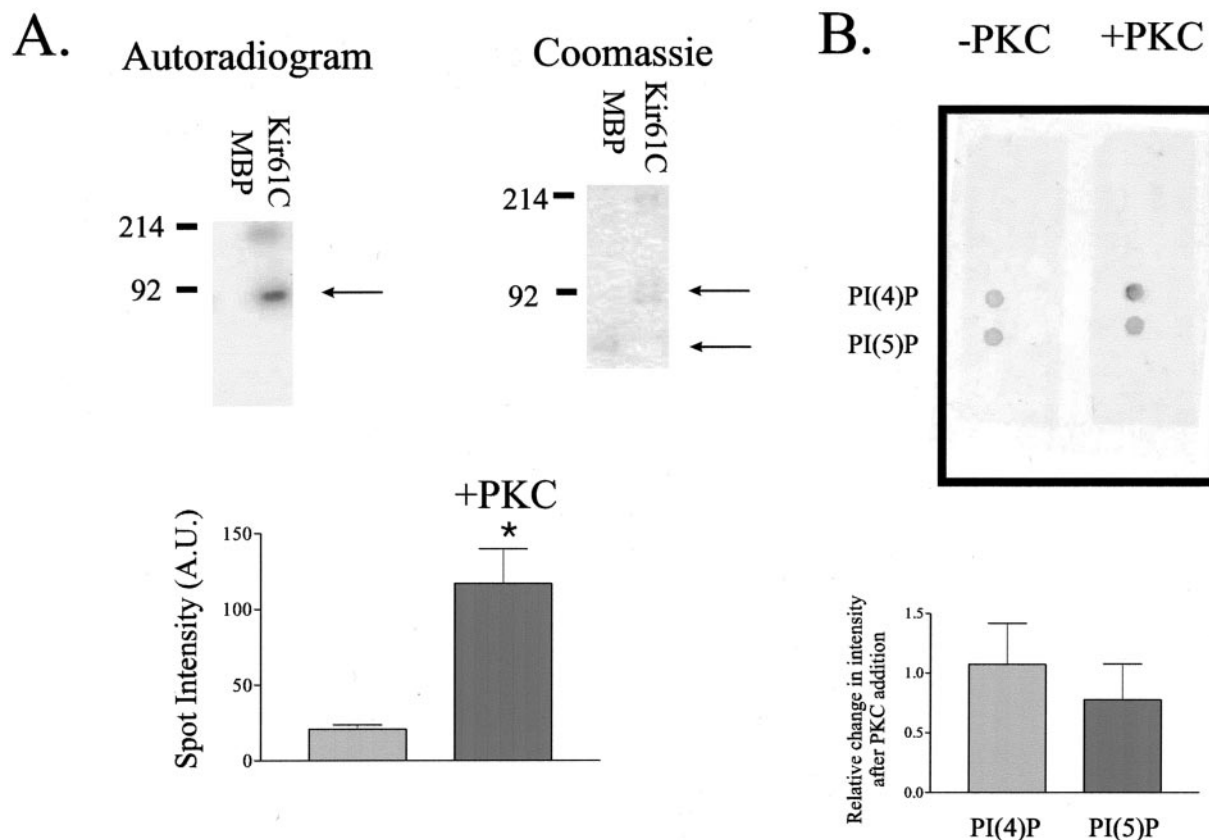
Figure 8. Phosphorylation by PKC of the C-terminus of Kir6.1 and the consequences for phospholipid binding. A, MBP-Kir61C, but not MBP, is phosphorylated by PKC. The bar chart shows quantification of the bands on the autoradiogram (n=5). *$P<0.05$. B, Phosphorylated protein was still able to bind with high affinity to PIP strips (as shown in Figure 7). The bar graph shows quantification (n=5).

Kir6.1 C-terminus binds with higher affinity to the phospho-inositol species, such as PtdIns(4)P than any others, including PtdIns(4,5)P_2, and may explain why the PtdIns(4,5)P_2 antibody did not affect whole-cell currents and why PtdIns(4,5)P_2 reactivated SUR2B/Kir6.2 better in the presence of poly-L-lysine. Studies to date show that the whole complex of Kir6.2 can bind to a range of inositol phospholipids[37] and that inward rectifiers have distinct phosphoinositide preferences at the functional level.[36]

Thus, our data show evidence of distinct inhibitory mechanisms, accounting for regulation of the highly homologous Kir6.1 and Kir6.2. The elucidation of residues and domains underlying these phenomena will contribute significantly to their understanding, and we are currently pursuing such studies.

Acknowledgments

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