Mechanism of PLC-Mediated Kir3 Current Inhibition

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ABSTRACT

A large number of ion channels maintain their activity through direct interactions with phosphatidylinositol bisphosphate (PIP_2). For such channels, hydrolysis of PIP_2 causes current inhibition. It has become controversial whether the inhibitory effects on channel activity represent direct effects of PIP_2 hydrolysis or of downstream PKC action. We studied Phospholipase C (PLC)-dependent inhibition of G protein-activated inwardly rectifying K⁺ (Kir3) channels. By monitoring simultaneously channel activity and PIP_2 hydrolysis, we determined that both direct PIP_2 depletion and PKC actions contribute to Kir3 current inhibition. We show that the PKC-induced effects strongly depend on PIP_2 levels in the membrane. At the same time, we show that PKC destabilizes Kir3/PIP_2 interactions and enhances the effects of PIP_2 depletion on channel activity. These results demonstrate that PIP_2 depletion and PKC-mediated effects reinforce each other and suggest that both of these interdependent mechanisms contribute to Kir3 current inhibition. This mechanistic insight may explain how even minor changes in PIP_2 levels can have profound effects on Kir3 activity. We also show that stabilization of Kir3/PIP_2 interactions by Gβγ attenuates both PKC and Gq-mediated current inhibition, suggesting that diverse pathways regulate Kir3 activity through modulation of channel interactions with PIP_2.

INTRODUCTION

The activity of an impressive number of ion channels has been shown to depend on phosphatidylinositol-4,5-bisphosphate (PIP_2) (reviewed in ref. 1). Hydrolysis of PIP_2 by stimulation of Phospholipase C (PLC) enzymes either via G protein signaling pathways (PLCβ) or growth factor receptors (PLCγ) results usually in inhibition of activity. The mechanism by which PLC activation leads to current inhibition has been attributed to either direct PIP_2 depletion from the channel, or PKC-mediated effects. Kir3 channels are G-protein regulated, K⁺ selective, inwardly rectifying channels expressed in cardiac, neuronal and endocrine cells, where they play an important role in regulating membrane excitability. These channels are regulated by direct binding of the G-protein-βγ subunits and, like all members of the Kir channel family, are dependent on PIP_2 for activity. Kir3 channels are regulated by an array of hormones and neurotransmitters, which include Acetylcholine (ACh), γ-aminobutyric acid (GABA), morphine, substance P and serotonin. The vast majority of these regulators act through G-protein coupled receptors (GPCRs) that couple to Gα, Gs and Gq/11 G proteins. Regulation of Kir3 in heterologous expression systems through growth factor receptors, i.e., Epidermal Growth Factor Receptor (EGFR), has also been shown to occur. In addition to being dependent on Gβγ and PIP_2, Kir3 channels are mechanosensitive and are also regulated by Na⁺, Mg^2+, ATP, extracellular K⁺, changes in pH and redox potential and a number of kinases, such as Protein Kinase A (PKA) and Protein Kinase C (PKC), and phosphatases such as Protein Phosphatase 2a (PP2a) and Protein Phosphatase 1 (PP1) (reviewed in refs. 19, 20 and 24).

Activation of Kir3 channels is now widely accepted to be due to the activation of Gi-proteins and Gβγ. While the mechanism of the inhibition of these channels is a topic of debate. As with other PIP_2-dependent channel families, both depletion of PIP_2 and downstream activation of PKC have been shown to be involved in Kir inhibition but the exact roles of PIP_2 and PKC and the extent to which they contribute to current inhibition remains controversial. Moreover, PKC activation independently of PIP_2 hydrolysis has been shown by several laboratories to lead to current inhibition, but the mechanism of PKC-mediated current inhibition remains controversial, despite extensive efforts.
In the present work we studied the roles of direct PIP₂ depletion and PKC action and addressed the interdependent contributions of these mechanisms in Kir3 current inhibition. Our findings indicate that both direct PIP₂ depletion from the channels and PKC effects make significant contributions towards Kir3 current inhibition. Moreover, our results suggest that PKC, once activated, decreases the apparent affinity of Kir3 for PIP₂ thus destabilizing Kir3/PIP₂ interactions and amplifying the effect of PIP₂ depletion on the channel. We show that the extent of the PKC-mediated effects depends on the membrane PIP₂ levels, in that they are enhanced by low PIP₂ levels. Our study suggests that the PLC-mediated inhibition of Kir3 channels involves two interdependent mechanisms: direct PIP₂ depletion from the channel and PKC activation and subsequent action. This mechanistic insight may explain how even minor changes in PIP₂ levels can have profound effects on Kir3 activity. Moreover, our results indicate that stabilization of Kir3/PIP₂ interactions with Gβγ attenuates both PKC and Gq-mediated current inhibition, suggesting that diverse pathways regulate Kir3 activity through modulation of channel interactions with PIP₂.

**MATERIALS AND METHODS**

Electrophysiological measurements in HEK 293 cells. Whole-cell recordings in HEK-293 cells were obtained with a 3900A Dagan amplifier. Ramp protocols from -80 mV to +80 mV were used to acquire currents. Data were digitized, recorded and analyzed at -80 mV using pClamp 9 software (Axon Instruments, CA). The pipette solution contained (in mM): 107 KCl, 1.2 MgCl₂, 1.0 CaCl₂, 2.0 MgATP, 0.3 NaGTP, 5.0 EGTA, 10 HEPS/KOH, pH 7.2. FVPP solution, containing (in mM) 5 NaF, 3 Na₃VO₄ and 10 Na₂P₂O₇, was added to the pipette solution.

The external solution contained (in mM): 140 KCl, 1.2 MgCl₂, 1.0 CaCl₂, 10 HEPS/KOH, pH 7.2. Electrode resistances were 3–4 MΩ. "zero or 0" current (as represented in figures by the dotted line) indicates current after application of BaCl₂ (1 mM) to block channel activity.

HEK-293 cells were cultured in DMEM (Fisher Scientific), supplemented with penicillin, streptomycin and 10% fetal calf serum (Fisher Scientific). Cells were transfected with the cDNAs of interest using the Effectene reagent (Qiagen). For experiments performed 24–72 hrs after transfection by examining cells plated on coverslips on a Nikon inverted microscope (Eclipse TE2000-S) with an oil-immersion 40x objective (CFI S Fluor 40x oil: N.A. 1.30; Nikon, Japan).

Conventional epifluorescence measurements were performed on individual whole cells. An area of interest was manually selected with shutters on photodiodes that adjust position and size of the field aperture to restrict illumination to a specific region thus allowing the measurements to be taken on individual cells (TILL Photonics Planegg, Germany). The CFP (donor fluorophore) was excited at 430 nm and the emissions of both the CFP and the YFP fluorophores were simultaneously monitored by utilizing dichroics, 455DCLP (Chroma Technology Corp., Rockingham, VT) and DCLP 4400 (TILL, Photonics, Planegg, Germany) and emission filters 480 ± 10 nm and 535 ± 15 nm (TILL Photonics, Planegg, Germany). Polychrome IV (TILL, Photonics, Planegg, Germany), a monochromatising device with an integrated Xenon lamp, was used as a light source. Photodiode detector heads were used to convert the photo current at two different emission wavelengths into voltage signals. Signals were integrated, digitized and collected using pClamp 9 software. Data were analyzed using Origin software (see later in Data analysis section). The ratio of YFP to CFP emission intensities (535/480) was calculated and used as a measure of FRET.

In order to establish a coherent comparison between measurements and to correct for possible bleed-through, the sensitized emission offset and FRET signals. (Fc) were calculated for each cell before (Fc-AA) and after (Fc-DA) agonist application.49-51 ΔF was calculated for each cell and FRET change was expressed as ΔF/ΔF (Fc-AA):

\[
F_c = \frac{I_{DA} - a_{AA} \cdot d_{DD}}{I_{DA} - a_{AA} \cdot d_{DD}}
\]

\[
\Delta F = F_{(c,D2)} - F_{(c,A1)} = I_{DA(D2)} - a_{AA(D2)} \cdot d_{DD(D2)} - (I_{DA(A1)} - a_{AA(A1)} \cdot d_{DD(A1)}
\]

where F_{(c,A1)} and F_{(c,D2)} are fluorescent intensities before and after agonist application; I_{DA} fluorescent intensity at 535 nm following excitation of donor at 430 nm using the FRET filter set (for detailed description of set see above); I_{AA} fluorescent intensity at 535 nm following excitation of acceptor at 505 nm in an acceptor-only
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Molecule using the FRET filter set (for detailed description of filter set see above); \(I_{DD}^{fluorescent\ intensity\ at\ 480\ nm\ following\ excitation\ of\ donor\ at\ 430\ nm\ in\ a\ donor-only\ specimen\ using\ the\ FRET\ filter\ set\ (for\ detailed\ description\ of\ filter\ set\ see\ above).\) The normalized parameters "a" and "d" account for the percentage of CFP and YFP bleed-through respectively and are exclusively dependent on the specific characteristics of our optical system. Control experiments proved \(I_{DD}^{fluorescent\ intensity\ at\ 480\ nm\ following\ excitation\ of\ donor\ at\ 430\ nm\ in\ a\ donor-only\ specimen\ using\ the\ FRET\ filter\ set\ (for\ detailed\ description\ of\ filter\ set\ see\ above).\) and \(I_{DD}^{fluorescent\ intensity\ at\ 480\ nm\ following\ excitation\ of\ donor\ at\ 430\ nm\ in\ a\ donor-only\ specimen\ using\ the\ FRET\ filter\ set\ (for\ detailed\ description\ of\ filter\ set\ see\ above).\) to be negligible for the combination of filters used in the system (data not shown; for a detailed description of the filter set see above). \(I_{AA}^{fluorescent\ intensity\ at\ 480\ nm\ following\ excitation\ of\ donor\ at\ 430\ nm\ in\ a\ donor-only\ specimen\ using\ the\ FRET\ filter\ set\ (for\ detailed\ description\ of\ filter\ set\ see\ above).\) and \(I_{AA}^{fluorescent\ intensity\ at\ 480\ nm\ following\ excitation\ of\ donor\ at\ 430\ nm\ in\ a\ donor-only\ specimen\ using\ the\ FRET\ filter\ set\ (for\ detailed\ description\ of\ filter\ set\ see\ above).\) did not vary significantly in our experiments (data not shown), moreover "a" was approaching "0" using the filter sets described above. The relative decrease of the net FRET value \(\Delta Fc/Fc\) for each measurement was expressed as a percentage of the initial FRET signal \(Fc_{(t1)}\).

Simultaneous measurements of whole-cell currents and changes in FRET in HEK293 cells. In order to study how PIP_{2} hydrolysis correlated with Kir3 channel activity, we monitored changes in whole-cell currents simultaneously with FRET changes. We overexpressed fluorescently tagged PH-domains of PLC\(\delta\), together with Kir3 and M1R and monitored simultaneous changes in current and FRET on single cells; for a detailed description of current/fluorescence measurements see above.

Macropatch experiments in Xenopus oocytes. Excised macropatch experiments were performed with an EPC-7 patch-clamp amplifier. Ramp protocols from -80 mV to +80 mV were used to acquire currents. Data were digitized, recorded and analyzed at -80 mV using pClamp software (Axon Instruments, CA). The pipette solution contained (in mM): 96 KCl, 1.8 CaCl_{2}, 1 MgCl_{2}, 1 NaCl, and 10 Hepes, pH 7.4. The bath solution contained (in mM): 76 KCl, 20 mM NaCl, 5 EGTA, and 10 HEPES, pH 7.4. Electrode resistances were 2–4 M\(\Omega\).

Kir3.1 and Kir3.4 constructs in the pGMHE plasmid vector were linearized with N\(\Phi\)el, and cRNAs were transcribed in vitro using the "message machine" kit (Ambion, Austin, TX). Xenopus oocytes were surgically removed, dissociated by collagenase treatment, and microinjected with the desired cRNA. Experiments were performed 24–72 hrs after injection.

Data analysis. ORIGIN (Microcal, Amherst, MA) was used for data analysis and statistics. Data in all figures were expressed as mean ± standard error of the mean. Statistical significance was evaluated by One-Way ANOVA analysis and was represented as: *\(p \leq 0.05\); **\(p \leq 0.01\); ***\(p \leq 0.001\).

Chemicals. PMA, BIS and wortmannin were obtained from Sigma-Aldrich (USA). The catalytic subunit of PKC\(\delta\) was purchased

Figure 1. PIP_{2} hydrolysis as monitored by translocation of fluorescently-tagged PH-domains of PLC\(\delta\): comparison between confocal and FRET measurements. (A). Confocal images taken before and after application of ACh to cells transfected with eYFP-PH-domain and Muscarinic Receptor Type 1. Left panel shows illustrative frames taken at points (a) before ACh application, (b) during ACh application, (c) during ACh application when steady state was reached, (d) during wash out of ACh. Right panel, a representative time course showing changes in fluorescence in response to ACh application (change in fluorescence is represented here by a ratio of membrane to cytoplasmic fluorescence, \(\frac{Fm-Fc}{Fc}\)). (B) Representative traces showing changes in fluorescence intensity in response to ACh. Left panel, changes in CFP emission as monitored at 480 nm (blue trace); changes in YFP emission as monitored at 535 nm (yellow trace); changes in the ratio of YFP over CFP and calculated 535 nm/480 nm (red trace). Right panel, a representative trace showing changes in the FRET time course (ratio of 535 nm/480 nm) before during and after ACh application. Fluorescence was monitored on a single cell. HEK 293 cells were transfected with M1R, the eCFP-PH domain and the eYFP-PH domain of PLC\(\delta\). (C) Comparison of confocal and FRET parameters. Top panel, kinetics of PH-domain translocation as measured by confocal and compared to kinetics in FRET change \(t_{1/2, sec}\); mean for confocal data 1.76 ± 0.56 (n = 5); mean for FRET data 2.30 ± 0.29 (n = 8). Bottom panel, magnitude of fluorescence change measured by confocal as compared to changes in FRET \(\frac{(Fm-Fc)}{Fc}\); vs \(\frac{\Delta Fc}{Fc_{(t1)}}\); mean for confocal data 20.0 ± 5.4 (n = 5); mean for FRET data 19.9 ± 5.3 (n = 8).
In order to clarify the donor CFP resulted in energy transfer to the acceptor YFP giving PH-domains concentrated in the plasma membrane and excitation of with membrane currents. Under unstimulated conditions, the tagged changes in FRET as a means to assess PIP$_2$ (e.g., 4; Supplementary movie).

Results

**PIP$_2$ hydrolysis underlies Kir3 inhibition.** In order to clarify the roles of PIP$_2$ depletion and PKC actions in Kir3 current regulation, we used a technique that allowed us to monitor channel activity simultaneously with PIP$_2$ hydrolysis. In single cells, we used electrophysiology to monitor whole-cell Kir3 currents at the same time we monitored PIP$_2$ hydrolysis by measuring changes in Fluorescent Resonance Energy Transfer (FRET) between fluorescently tagged PH-domains of PLC$_\delta$.

The PH-domain of PLC$_\delta$, labeled with the Green Fluorescence Protein (GFP) has been used extensively to assess PLC activation and PIP$_2$ hydrolysis in living cells by monitoring changes in GFP fluorescence at the cell surface versus the cytoplasm. We used PH-domains of PLC$_\delta$ tagged with either of two variants of GFP, CFP (cyan) and YFP (yellow) and transfected them into HEK293 cells. Figure 1A shows ACh-mediated translocation and recovery of the YFP-tagged PH domain of PLC$_\delta$, in response to activation of Muscarinic Receptor Type 1 (M1R) by ACh monitored through confocal microscopy. The ACh-induced translocation of the PH domain to the cytoplasm displayed rapid kinetics relative to its recovery in the plasma membrane, consistent with previous reports (e.g., 4; Supplementary movie).

We proceeded to monitor through use of an inverted microscope changes in FRET as a means to assess PIP$_2$ hydrolysis simultaneously with membrane currents. Under unstimulated conditions, the tagged PH-domains concentrated in the plasma membrane and excitation of the donor CFP resulted in energy transfer to the acceptor YFP giving rise to basal FRET levels (Fig. 1). Upon PLC activation, the CFP- and YFP-tagged PH-domains were redistributed to the cytoplasm resulting in a decrease in FRET representing a drop in membrane PIP$_2$. Even though, the use of fluorescent PH-domains as a measure of membrane PIP$_2$ levels has been debated, work by van der Wal and colleagues has provided direct evidence that PLC-mediated changes in fluorescence indeed represent a decrease in membrane PIP$_2$ rather than an increase in cytosolic IP$_3$. We compared changes in FRET between fluorescently tagged PH-domains to changes in confocal signals representing translocation of a PH-domain in response to ACh application to HEK293 cells expressing M1R. Figure 1B demonstrates ACh-induced changes in whole-cell FRET as monitored by changes in fluorescence emissions of CFP (blue trace, measured at 480 nm) and YFP (yellow trace, measured at 535 nm). FRET is calculated as a ratio of YFP/CFP emissions (red trace, calculated as 535 nm/480 nm). ACh binding to M1R leads to activation of PLC$_\beta$ causing PIP$_2$ hydrolysis that is reflected by either translocation of PH-domain (as in the case of the confocal experiment, Fig. 1A) or by measuring a decrease in the FRET signal (Fig. 1B red trace); stimulus-induced changes in FRET for each cell were expressed as % change in calculated fluorescence $\Delta Fc/Fe_{(0)}$ (for a detailed description of the calculations see the Materials and Methods section). The two methods of monitoring PIP$_2$ hydrolysis (i.e., confocal versus FRET) were compared in terms of the kinetics of translocation ($t_{1/2}$) and the change in fluorescence magnitude. In the case of the confocal experiment, the change in the ratio of the membrane to cytoplasmic fluorescence was calculated; in the case of FRET, the change in whole-cell FRET was calculated (for a detailed description of these calculations see the Materials and Methods section). Both measurements provided comparable results (Fig. 1C).

The fluorescent signal recovers slowly following removal of ACh, representing the slow process of PIP$_2$ resynthesis (Fig. 1A and B). Equal concentrations of CFP and YFP-PH-domain cDNA were used in all experiments described in this work; changes in ratios of CFP to YFP (CFP10:YFP1 and vise versa) did not result in significant changes in FRET (Supplementary Fig. 1A). Furthermore, when CFP-tagged PH-domain was expressed alone, no change in FRET was observed during ACh application (Supplementary Fig. 1B).

Since the FRET changes reported PIP$_2$ hydrolysis in a manner similar to the confocal PH translocation experiment, we proceeded to work with an inverted microscope and record simultaneously PIP$_2$...
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hydrolysis and ionic currents. Simultaneous ionic current and FRET measurements made it possible for us to compare the kinetics of Kir3 inhibition to changes in PIP_{2} hydrolysis. We used the Gi-coupled Muscarinic Type 2 Receptor (M2R) or δ opioid receptor (δOR) to activate Kir3 currents and the Gq-coupled Muscarinic Type 1 Receptor (M1R), to inhibit them. Simultaneous measurements of current (black traces) and FRET (red traces) in response to ACh in cells expressing the Kir3 channel and PH-domains are shown in Figure 2A. ACh-induced current inhibition correlated well with FRET changes in cells expressing M1R together with M2R (left panel). In contrast, in cells expressing M2R alone (right panel) no ACh-induced current inhibition or FRET changes were observed.

We next used the δOR to activate Kir3 and M1R to inhibit it, in order to separate current activation from inhibition and thus more clearly correlate kinetics of current inhibition with those of PIP_{2} hydrolysis (Fig. 2B). The times required for half maximal current inhibition (black bar) and FRET changes (red bar) were quite similar consistent with the interpretation that hydrolysis of PIP_{2} underlies current inhibition (Fig. 2B, right panel).

Direct activation of PKC results in channel inhibition bypassing PIP_{2} hydrolysis. PKC stimulation by phorbol esters, acting independently of PLC activation, is known to cause Kir3 inhibition but the mechanism of PKC action on channel activity remains unknown. Zhang and coworkers reported based on their results that the PKC activator Phorbol 12-myristate 13-acetate (PMA) itself induces hydrolysis of PIP_{2} and proposed that PKC might inhibiting the Kir3 currents by depleting PIP_{2}. In order to evaluate this hypothesis, we tested different concentrations of PMA for their ability to inhibit basal Kir3 currents and to cause PIP_{2} hydrolysis, as monitored by FRET changes. 100 nM PMA caused a maximal inhibition of basal Kir3 currents (Fig. 3A) without causing a change in FRET as compared to M1R stimulation by ACh (Fig. 3B). Thus our results suggest that on the time scale that PMA inhibited Kir3, it did not alter either PLC activity or PIP_{2} hydrolysis. These experiments suggest that unlike prior interpretations, PKC exerts its effects on Kir3 independently of causing PIP_{2} hydrolysis.

Both direct PIP_{2} depletion and PKC action contribute to receptor-mediated inhibition of Kir3. We next asked whether PKC activation alone could account for all of the M1R-mediated Kir3 inhibition. In order to address this question, we stimulated the M1R with ACh following application of saturating PMA concentrations (to fully activate PKC) and asked whether any additional inhibition of basal Kir3 activity could be attributed to PIP_{2} depletion.

Figure 4A shows the effect of ACh, as it was applied following PMA, on simultaneous recordings of whole-cell currents (in black) and PIP_{2} hydrolysis (in red), as monitored by changes in FRET. As before (Fig. 3), a saturating concentration of PMA (100 nM) inhibited basal channel activity without causing PIP_{2} hydrolysis. Subsequent
application of ACh resulted in an initial current activation (presumably due to nonspecific stimulation of Kir3 channel by Gbg coupled to the promiscuous M1R)\textsuperscript{14} that was followed by an inhibitory component that saturated at levels beyond those resulting from the PMA application alone. This inhibition of basal Kir3 currents tightly correlated with changes in FRET indicating PIP\textsubscript{2} hydrolysis. These results suggest that receptor-mediated inhibition of Kir3 currents by ACh involves direct PIP\textsubscript{2} depletion from the channel.

In order to determine whether the results obtained with activation of M1R that couples to PLC\textsubscript{b} (Fig. 4A) could be generalized to other PLC-coupled receptors that signal independently of G proteins, we studied the effect of the Epidermal Growth Factor Receptor (eEGFR) that couples to PLC\textsubscript{G} and was previously shown to inhibit Kir3 currents.\textsuperscript{2} Stimulation of EGFR (with 100 ng/mL EGF) following application of a saturating PMA concentration (100 nM) resulted in additional inhibition of basal Kir3 activity presumably through PIP\textsubscript{2} depletion (Fig. 4B). This result is consistent with the effect seen with M1R stimulation. Taken together these results suggest that in the background of saturating PKC activity, Kir3 inhibition resulting from stimulation of either PLC\textsubscript{b} through M1R or PLC\textsubscript{G} through EGFR involves direct PIP\textsubscript{2} depletion from the channel.

To further separate and quantify the effects of PKC and direct PIP\textsubscript{2} depletion on Kir currents, we used a specific PKC inhibitor, bisindolylmaleimide II (BIS), to block PKC-mediated effects. We first determined the concentration of BIS, which could fully block the maximal PMA-induced effect on Kir3. 100 nM BIS completely abolished the effect of saturating PMA concentrations on Kir3; yet, ACh application resulted in a PKC-independent inhibition of Kir currents, presumably due to the direct effects of PIP\textsubscript{2} depletion from the channel (Fig. 4C). We thus used 100 nM BIS to study the
PKC-independent component of ACh-mediated Kir3 inhibition. Simultaneous measurements of whole-cell current and FRET changes show that 100 nM BIS attenuated significantly the ACh-induced inhibition of Kir3 currents but did not affect FRET (Fig. 4D). These results suggest that the ACh-induced inhibition of Kir3 currents is comprised of two components: one due to PKC activation (20–30%), while the other due to direct PIP$_2$ depletion (40–50%).

PKC action and PIP$_2$ depletion effects on Kir3 currents are interdependent. PKC-mediated effects are enhanced by low membrane PIP$_2$ levels. We next sought to investigate whether direct PIP$_2$ depletion and PKC activation influenced one another during Kir3 inhibition. We first tested whether decreased or increased PIP$_2$ levels would influence PKC-mediated channel inhibition. To decrease PIP$_2$ levels, we used wortmannin, a fungal metabolite, which at µM concentrations inhibits certain PI4K isoforms, thus decreasing whole-cell PIP$_2$ levels (e.g., refs. 4 and 7). Wortmannin (26 µM) treatment enhanced the PMA-mediated inhibition of Kir3 currents, suggesting that PKC has a more pronounced effect on channel activity when PIP$_2$ levels are low (Fig. 5A and B). To increase PIP$_2$ levels, we first overexpressed PIP-5 kinase (PIP5K) (Fig. 5A–C). PIP5K is an enzyme that phosphorylates PI(4)P at position 5 of the inositol ring to make PIP$_2$, and its overexpression has been widely used to study the effects of increasing PIP$_2$ levels in cells (e.g., refs. 7 and 39). Alternatively, we utilized diC$_8$ PIP$_2$ in order to directly increase PIP$_2$ levels in the cell (Fig. 5A). diC$_8$ PIP$_2$ is a soluble synthetic analog of PIP$_2$, which has been used to increase intracellular PIP$_2$ concentrations through the patch pipette. Both PIP5K overexpression and inclusion of 200 µM diC$_8$ PIP$_2$ in the
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recording pipette attenuated the PMA-induced inhibition of Kir3 currents (Fig. 5A), indicating that the PKC-mediated inhibition of Kir3 is significantly attenuated at high membrane PIP$_2$ levels. Taken together, these results suggest that the PKC-mediated effects are inversely dependent on PIP$_2$ levels in the membrane.

We next sought to examine how membrane PIP$_2$ levels affected Gq-mediated inhibition of Kir3 currents. The ACh-induced, like PMA-mediated, inhibition of Kir3 currents was enhanced in cells treated with wortmannin and attenuated in cells overexpressing PIP5K (Fig. 5B), suggesting that M1R-mediated Kir3 inhibition is also sensitive to changes in membrane PIP$_2$ levels. Furthermore, these results suggest that the extent of receptor-mediated inhibition of Kir3 is directly related to the interdependence between the PKC and PIP$_2$ depletion effects.

PIP5K overexpression resulted in a higher apparent localization of the PH domains at the plasma membrane, suggesting increased membrane PIP$_2$ levels (Fig. 5C). Global PIP$_2$ hydrolysis, as monitored through confocal microscopy (not shown) or FRET changes (Fig. 5D) by the translocation of fluorescent PH-domains, remained unchanged, regardless of the relative membrane PIP$_2$ levels. Thus, there were no differences in FRET changes in response to ACh application among cells treated with wortmannin (i.e., decreased PIP$_2$ levels), to those overexpressing PIP5K (i.e., increased PIP$_2$ levels) as compared to control cells. Thus, since PMA- and ACh-induced inhibition inversely depended on the levels of PIP$_2$ and differences in PIP$_2$ hydrolysis could not account for this dependence, we hypothesized that PKC action depended on the levels of PIP$_2$ in the plasma membrane. In order to further test this hypothesis, we tested the contributions of PKC towards ACh-induced inhibition under low and high PIP$_2$ levels. Using BIS to control PKC activity, we showed that ACh inhibition was highest under low PIP$_2$ conditions only when PKC was active (Fig. 5E). Interestingly, in cells pretreated with wortmannin and BIS (low PIP$_2$, PKC inhibited) the extent of ACh-induced inhibition was similar to that seen in cells overexpressing PIP5K (high PIP$_2$, Fig. 5E), suggesting that in the absence of PKC activity Kir3 did not sense as much the drop in membrane PIP$_2$ levels. These results argue that PKC activation sensitized Kir3 to inhibition when PIP$_2$ levels became low.

PKC decreases the apparent affinity of Kir3 for PIP$_2$. In order to further test this hypothesis, we studied whether PKC activation has an effect on Kir3 regulation by PIP$_2$. We first tested whether PKC affects channel inhibition through PIP$_2$ depletion, by measuring
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Figure 7. Effect of Gi coupled receptors and Gβγ on PKC and Gq-mediated inhibition of Kir3. (A) Representative traces and the summary data of PMA-induced inhibition of Kir3 currents in control cells or following activation of δ opioid receptor (δOR) by deltorphin II or in cells overexpressing Gβγ. % inhibition of basal currents by PMA: mean = 21.7 ± 1.4 (n = 3); % inhibition of deltorphin II-induced currents in cells expressing δOR: mean = 1.0 ± 1.0 (n = 3); % inhibition of basal currents in cells overexpressing Gβγ: mean = 2.0 ± 1.2 (n = 3). Dotted line and “0” represent “zero” current. HEK293 cells were transfected with Kir3, indicated receptors or Gβγ. (B) Representative traces and the summary data of the ACh-induced Kir3 inhibition in the presence of M1R alone (control cells), in the presence of stimulated δ opioid receptor (in these cells deltorphin II was applied prior to ACh application to activate δOR; we continued to apply deltorphin II together with ACh during the experiment); or in cells overexpressing Gβγ. % inhibition of basal currents by ACh mean: 77.1 ± 5.1 (n = 4); % inhibition of deltorphin II-induced currents in cells expressing δOR: mean = 39.3 ± 8.9 (n = 6); % inhibition of basal currents in cells overexpressing Gβγ mean: 46.8 ± 8.4 (n = 6). Ibasal stands for basal current; Idelt II stands for current activated by deltorphin II. Saturating concentrations of ACh (5 μM) and deltorphin II (1 μM) were used to obtain maximally activated currents. Dotted line and “0” represent “zero” current. HEK293 cells were transfected with Kir3, M1R and either δOR or Gβγ.

M1R-mediated inhibition under conditions of either fully activated PKC (pretreatment with 100 nM PMA) or fully inhibited PKC (pretreatment with 100 nM BIS). The ACh-induced inhibition of Kir3 currents was greater in the presence of PMA (PKC active) than in the presence of BIS (PKC inactive) (Fig. 6A). Neither PMA nor BIS had an effect on whole-cell PIP2 hydrolysis, as monitored by FRET (Fig. 6A, right panel), consistent with our earlier results (Fig. 4). These results further suggested that PKC activation sensitizes Kir3 to inhibition by PIP2 depletions.

We next proceeded to directly address whether PKC affected the apparent affinity of Kir3 channel for PIP2. We tested the effects of PIP2 in inside-out (i/o) macropatch recordings of Kir3 activity by using different concentrations of diC8 PIP2 before and after bath perfusion of the inside surface of the macropatch with the catalytic subunit of PKCδ together with ATPγS. The PKCδ isoform was recently shown by Brown et al.18 to play a role in Kir3 channel regulation. Figure 6B shows representative traces of current activation by different concentrations of PIP2 in the same membrane patch before and after application of the PKCδ catalytic subunit. The catalytic subunit of PKCδ decreased the apparent affinity of the Kir3 channel for PIP2 as demonstrated by the rightward shift in the PIP2 dose-response curves (Fig. 6C). In control experiments, where instead of PKCδ we perfused Kir3 expressing macropatches with the bath solution (see Materials and Methods section), the amplitudes of the PIP2-induced Kir3 currents were the same during the first and second applications of diC8 PIP2 (data not shown).

We also attempted to test whether treating oocytes with the PKC inhibitor BIS would further accentuate the PKCδ-induced rightward shift of the PIP2 dose-response curve, but we were unable to obtain long-lived macropatches under these conditions.

Gβγ released following activation of Gi-coupled receptors attenuates the extent of Gq-mediated channel inhibition. Gβγ was previously shown to stabilize Kir3/PIP2 interactions, thus leading to increased channel activity.23,41 In order to investigate whether stabilizing Kir3/PIP2 interactions with Gβγ would attenuate the Gq-mediated effects, we first addressed whether Gβγ affected the PKC-mediated inhibition of Kir3 channels. We expressed either the δ opioid receptor (δOR) or exogenous Gβγ together with Kir3 and measured the PMA-induced inhibition of basal currents. Gβγ, either released following stimulation of δOR or overexpressed (+Gβγ), greatly attenuated the PMA-induced inhibition of Kir3 currents (Fig. 7A). These results suggest that stabilizing channel-PIP2 interactions retards channel inhibition by PKC. We next studied the effect of overexpressed Gβγ on the Gq-mediated channel inhibition. The M1R-mediated inhibition of Kir3 was attenuated following activation of δOR or in the presence of overexpressed Gβγ (Fig. 7B), results that further support the conclusion that the PKC component of the Kir3 inhibition is greatly dependent on channel/PIP2.
interactions. These results are consistent with the interpretation that Gβγ decreases the extent of Gq-mediated inhibition of Kir3 through strengthening channel/IP3 interactions,\textsuperscript{5,23,41} thus leading to an attenuation of the PKC-mediated effects on channel activity.

Overall these results suggest that there is interplay between the activation pathway (via Gi-coupled receptors and Gβγ) and the inhibitory pathway (via receptors activating PLC and PKC) at the level of IP3-mediated regulation of Kir3: with Gi and Gβγ strengthening Kir3/IP3 interactions and PKC weakening them.

**DISCUSSION**

The mechanism of PLC-mediated inhibition of Kir3 currents, with regards to the contribution of direct IP3 depletion versus effects of downstream IP3 hydrolysis products, has remained unclear despite considerable effort by several laboratories. Both IP3 and PKC have been shown to be involved in channel inhibition but their relative roles remain controversial. In an attempt to uncover the role of PKC in Kir3 inhibition by the Gq-coupled receptors, Hill and Peralta mutated five consensus PKC phosphorylation sites but found no change in the inhibition of Gβγ-stimulated currents.\textsuperscript{29} They concluded that the mutated sites were not representative of PKC phosphorylated sites. Since Gβγ greatly attenuates PMA-induced inhibition (Fig. 7), this conclusion is questionable. Mao and colleagues\textsuperscript{47} reported identification of critical PKC phosphorylation sites (Ser185 in Kir3.1 and Ser191 in Kir3.4) that fully controlled the PKC effects. Yet, neither Brown and colleagues nor our group have been able to fully reproduce these findings\textsuperscript{18} (also Logothetis lab, unpublished results). Despite the lack of definitive identification of all the PKC modified sites, Brown and colleagues proposed that the PKC-mediated inhibition was likely to be direct after demonstrating channel inhibition by purified PKC\textsubscript{δ} in excised patches.\textsuperscript{18} On the other hand, Zhang et al. proposed that PKC itself exerted its actions by causing IP3 hydrolysis.\textsuperscript{10} In fact, PKC has been shown to regulate PLC activity,\textsuperscript{42} and PKC activation by phorbol esters led to changes in IP3 levels after pretreating cells with PMA for at least 12 min.\textsuperscript{43} Nikolov and Ivanova-Nikolova postulated that PKC inhibits Kir3 by reducing the effectiveness of Gβγ on channel activation.\textsuperscript{52} Thus the mechanism of the PKC-mediated inhibition has remained unclear. Moreover, PKC activation alone could not account for receptor-mediated effects\textsuperscript{11,12} and the involvement of direct IP3 depletion from the channel during Kir3 inhibition could not be ruled out.

The present work further clarifies the mechanism underlying the PLC-mediated inhibition of Kir3 channels and elucidates the roles of PKC and IP3 in channel regulation. We demonstrate rapid effects of PMA on Kir3 activity without corresponding effects on IP3 hydrolysis; these results suggest that on the time scale of our experiments, PKC exerts its effects on Kir3 channels without causing changes in IP3 hydrolysis. In fact, our results indicate that both PKC action and direct IP3 depletion contribute to channel inhibition. Moreover, we show that these two mechanisms are interdependent: PKC is more effective in mediating channel inhibition when membrane IP3 levels are low (as in the case of IP3 hydrolysis); and PKC, once activated, destabilizes the interactions between Kir3 and IP3, thus sensitizing the channel to inhibition by IP3 depletion. This result is consistent with a recent finding by Thomas and colleagues showing decreased binding between anionic phospholipids and the C-terminus of Kir3.1 following phosphorylation by PKC.\textsuperscript{44}

Thus PKC and IP3, positively reinforce one another in order to maximize the effect of IP3 hydrolysis in inhibiting channel activity. Given previous reports, which suggest that there are no major changes in global IP3 levels in response to receptor stimulation,\textsuperscript{43} our findings could explain how even minor changes in IP3 levels can have significant local effects on Kir3 currents.

Our results further indicate that activation of Gi-coupled receptors leads to attenuation of both the Gq-coupled receptor and the PKC-mediated inhibition of Kir3 channels, presumably through the previously reported Gβγ-mediated stabilization of channel/IP3 interactions.\textsuperscript{53,41} These results suggest that activation of Gi-coupled receptors renders the channel less sensitive to regulation by Gq-coupled receptors and IP3 hydrolysis. Thus, both the Gi-mediated stimulatory pathway (via Gβγ-mediated stabilization of channel/IP3 interactions) and the Gq-mediated inhibitory pathway (via PKC-mediated destabilization of channel/IP3 interactions) regulate Kir3 activity through modulation of channel/IP3 interactions.

Furthermore, activation of Protein Kinase A (PKA), following stimulation of Gs-coupled receptors, was shown to attenuate the PLC-mediated inhibition of Kir3 as well as other representatives of the major K+ channel families (see accompanying paper).\textsuperscript{48} Taken together these results suggest a common mechanism of channel regulation by distinct signaling pathways that converge to regulate channel activity by modulating channel/IP3 interactions.

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