Protein Kinase A Modulates PLC-Dependent Regulation and PIP$_2$-Sensitivity of K$^+$ Channels

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ABSTRACT

Neurotransmitter and hormone regulation of cellular function can result from a concomitant stimulation of different signaling pathways. Signaling cascades are strongly regulated during disease and are often targeted by commonly used drugs. Crosstalk of different signaling pathways can have profound effects on the regulation of cell excitability. Members of all the three main structural families of potassium channels: inward-rectifiers, voltage-gated and 2-P domain, have been shown to be regulated by direct phosphorylation and Gq-coupled receptor activation. Here we test members of each of the three families, Kir3.1/Kir3.4, KCNQ1/KCNE1 and TREK-1 channels, all of which have been shown to be regulated directly by phosphatidylinositol bisphosphate (PIP$_2$). The three channels are inhibited by activation of Gq-coupled receptors and are differentially regulated by protein kinase A (PKA). We show that Gq-coupled receptor regulation can be physiologically modulated directly through specific channel phosphorylation sites. Our results suggest that PKA phosphorylation of these channels affects Gq-coupled receptor inhibition through modulation of the channel sensitivity to PIP$_2$.

INTRODUCTION

The activity of an impressive number of ion channels has been shown to depend on PIP$_2$ (reviewed in ref. 1). Potassium (K$^+$) channels are the largest and most diverse family of ion channels. They fall into three main groups based on their structural topology: 6 transmembrane helices or 6TM that include the voltage-gated channels, 4TM helices (i.e., the two-pore domain channels), and 2TM helices, such as the inward rectifiers (Kir). While all these channels are highly selective for K$^+$ over other ions, they exhibit distinct physiological and biophysical properties. Members from the three main potassium channel families have been shown to be PIP$_2$ sensitive.2-14 Several diverse forms of Kir channel modulation seem to exert their effects through modifying interactions of the channel with PIP$_2$.15,16 In addition, mutations of the inward rectifiers Kir2.1 and Kir1.1 that cause disruptions in channel-PIP$_2$ interactions result in Andersen’s and Bartter’s syndromes, respectively.17

In this study, we concentrated on the regulation of a representative member from each of the three main potassium channel families: the inward rectifier Kir3.1/Kir3.4 (K$_{ACh}$), the voltage-gated KCNQ1/KCNE1 (IKs) and the two-pore domain TREK1 (K2P2.1, KCNK2). For all three channels PIP$_2$ depletion has been shown to underlie inhibition of the channel by Gq stimulation.8,12,13 Following muscarinic type 2 (M2) acetylcholine receptor stimulation, the G protein $\beta\gamma$ subunits bind directly to K$_{ACh}$ and activate it.18 K$_{ACh}$ function is modulated by a large number of signaling molecules, including cytoplasmic ATP,19,20 Na$^+$,20 and unsaturated free fatty acids.21,22 K$_{ACh}$ channels are regulated by protein phosphorylation via PKA and dephosphorylation via PP2A.23,24 PKC also is known to inhibit K$_{ACh}$ currents.15,24-26 Kir3.1/Kir3.4 channels have been shown to form a complex with G protein-coupled receptor kinase, cAMP-dependent protein kinase, two protein phosphatases, PP1 and PP2A and the receptor for activated C kinase 1.27 IKs channels have been shown to be regulated by PKA28,29 and PKC28,30-32 phosphorylation and by increases in intracellular Ca$^{2+}$.33,34 TREK1 channels are known to be strongly regulated by PKA35,36 and also to be regulated by pressure, temperature and arachidonic acid.36,37

Our results show a crosstalk between channel phosphorylation by PKA and Gq/G11 regulation for all three channels tested. We show that the Gq/G11-mediated inhibition is critically but distinctly dependent on the channel PKA-dependent phosphorylation...
state. Moreover, our data suggest that the mechanism underlying the G-protein coupled receptor crosstalk is through direct channel phosphorylation altering interactions of the channel with PIP$_2$.

**MATERIALS AND METHODS**

**Molecular biology.** For oocyte experiments cDNA constructs were subcloned into either the plasmid vector pEXO (TREKI), pGEM-HE$^{38}$ (Kir3.1, Kir3.4, M1 and IP3 phosphatase) or its modified version PGM-sh (KCNQ1, KCNE1), to obtain optimal expression in *Xenopus* oocytes. The cDNA was linearized and cRNA was prepared using the Ambion mMESSAGE mACHINE T7 kit. Point mutations were produced by Pfu based mutagenesis with a QuikChange™ kit (Stratagene Inc., La Jolla, CA). HEK-293 cells were cultured in DMEM supplemented with 10% FBS. cDNAs for Kir3.1/Kir3.4 channels and M1/M2 receptors in the pcDNA3 vector were transfected using the Effectene (Qiagen) transfection reagent. For patch-clamp experiments, pEGFPN1 (a transfection marker) and the required cDNA were transfected at a ratio of 1:7. Experiments were performed 48–72 hr after transfection. For phosphorylation site predictions we used the NetPhos 2.0 server. The Kir3.1 S221 site predictions were performed 48–72 hr after transfection. For phosphorylation were transfected using the Effectene (Qiagen) transfection reagent. For patch-clamp experiments, pEGFPN1 (a transfection marker) and the required cDNA were transfected at a ratio of 1:7. Experiments were performed 48–72 hr after transfection. For phosphorylation site predictions we used the NetPhos 2.0 server. The Kir3.1 S221 residue received a score of 0.993.

**Cardiomyocyte preparation.** Cardiomyocytes were prepared as previously described with some modifications.$^{39}$ Guinea pigs (300–400 g) were anesthetized with 100 mg/kg sodium pentobarbital. Heparin (1 unit) was added to prevent blood clots. The heart was removed and immersed in ice-cold buffer (S1: 137 NaCl, 5.4 KCl, 1.2 MgSO$_4$, 7H$_2$O, 1.2 NaH$_2$PO$_4$, 15 Glucose, 20 HEPES). The aorta was cannulated and perfused in a Langendorff apparatus at 37°C with solutions bubbled with 100% O$_2$ at a flow rate of 4 ml/min. S1 was perfused for 5 min followed by digestion buffer (S2) for 10–12 min. S2 was S1 supplemented with 0.64 mg/ml type II collagenase (Worthington), 0.1% of BSA and 10 μM Ca$^{2+}$. Ventricles were removed, cut in small pieces, and further digested for 5–10 min at 37°C in S2 supplemented with 0.66 mg/ml creatine, 1.25 mg/ml taurine and 100 μM Ca$^{2+}$. Cells were mechanically dissociated and the procedure was repeated two times. Cells settled by gravity and were washed with S1 supplemented with 1% BSA, 1.25 mg taurine and with first 250 μM followed by 500 μM Ca$^{2+}$. The final wash was done in S1 with 1 mM Ca$^{2+}$. Cells were plated in cover slips coated with 10 μg/ml laminin and kept in M-199 medium supplemented with pen/strep. Recordings were made 2–48 hr after the isolation.

**Electrophysiology.** Oocytes were isolated from *Xenopus laevis* frogs (Nasco, Atkinson, WI) and injected with 0.5–15 ng of cRNA in 50 nl of sterile water. Macropatch measurements in the inside-out configuration were performed as described.$^{40}$ Electrodes for oocyte experiments contained in mM: 96 KCl, 1 CaCl$_2$, 1 MgCl$_2$, 5 HEPES, pH 7.4. For macropatch experiments the perfusion solution contained (in mM): KC1 96, EGTA 5, and HEPES 10, pH 7.4. Whole-oocyte currents were measured by conventional two-microelectrode voltage-clamp as previously described.$^{37}$ Bath solutions for Kir3.1/Kir3.4 experiments contained in mM: 96 KCl, 1 CaCl$_2$, 1 MgCl$_2$, 5 HEPES, pH 7.4. For KCNQ1/KCNE1 and TREKI experiments the bath solution contained in mM: 96 NaCl, 2 KCl, 1 CaCl$_2$, 1 MgCl$_2$, 5 HEPES, pH 7.4. For Kir3.1/Kir3/4 and TREKI experiments a ramp protocol from -150 to +100 mV was used (1 mV/ms) with a holding potential of 0 mV. For KCNQ1/KCNE1 experiments a holding potential of -80 mV was used followed by a voltage step to +40 mV for at least two seconds, unless otherwise specified.

Patch-clamp experiments on HEK-293 cells were performed in a solution containing, in mM, 140 KCl, 1 MgCl$_2$, 10 glucose, 10 HEPES, 1.8 CaCl$_2$, pH 7.4. Patch-clamp experiments on HEK-293 cells were performed using a HEKA amplifier and data were collected using the Pulse software (Heka, Germany).

Whole-cell recordings in ventricular myocytes were obtained with an Axopatch 200B amplifier (Axon Instruments). Four second depolarizing pulses to +40 mV from a -50 mV holding potential were used. A 50 ms prepulse to -40 mV was used to inactivate sodium currents. Pipettes with 2–5 megohms resistance were filled with (in mM): 110 potassium aspartate, 2 MgCl$_2$, 11 EGTA, 10 HEPES, 1 CaCl$_2$, and amphotericin 250 μg/ml (pH 7.3). The bath solution was (in mM): 132 NaCl, 5 KCl, 1 MgCl$_2$, 5 glucose, 5 HEPES, and 1 CaCl$_2$, pH 7.4. Nicardipine (10 μM) and E-4031 (5 μM) were added to inhibit Ca$^{2+}$ and IKr currents.

For single-channel experiments Kir3.1/3.4, 3 ng/oocyte of each of the channel subunits were injected and measured after 24-72 hours. Oocytes were incubated in OR2 medium (in mM: 82.5 NaCl, 2 KCl, 1 MgCl$_2$, 5 HEPES; pH 7.5 with NaOH) with 50 μM H89 for 1–2 hours before recordings. The bath solution contained (in mM): 91 KCl, KOH/HEPES each 5; 30 NaCl; 1 MgCl$_2$, pH 7.4. Pip$_2$-dC$_8$ (from Cayman Chemical Inc.) was prepared from 2 mM stocks. PKA catalytic subunit (from New England Biolab) 2,500 units/ml was applied with 2 mM ATP-γs. Single-channel activity was recorded when the membrane patches were held at -100 mV. Single-channel analysis was conducted with pClamp software (by Axon Instruments).

Error bars in the figures represent s.e.m. Each experiment shown or described was performed on a minimum of five oocytes from at least two frogs. The unpaired t-test was used to assess statistical significance.

**Phosphoprotein and total protein staining.** The C-termini of Kir3.1 (185-501) and Kir3.4 (184-419) were subcloned into pGEX-4T3 vector (Amersham Biosciences) and over-expressed in BL-21 (DE3) competent cells (Stratagene). GST-Kir3.1C and GST-Kir3.4C were purified with Glutathione Sepharose 4B (Amersham Biosciences). Phosphorylation of GST-Kir3.1C and GST-Kir3.4C by the PKA catalytic subunit (New England Biolabs) was performed at 30°C for an hour according to the manufacturer’s protocol. The reaction was stopped by adding SDS sample buffer and boiling for 5 min. Samples were loaded onto a NuPage Bis-Tris Gel (Invitrogen). Protein gel was stained first with Pro-Q Diamond Phosphoprotein Gel Stain (Molecular Probes) for the detection of protein phosphorylation, then with Novex Colloidal Blue Stain (Invitrogen) for the detection of total protein amount.

**Model of Kir3.1.** The crystallographic structure of the cytosolic domain of Kir3.1 (PDB accession number 1U4E) was combined with the transmembrane domain of KirBac3.1 (PDB accession number 1XL4). In order to connect the homology model of the N-terminus part that was based on the KirBac3.1 channel with the N-terminus part included in the structure of the cytosolic domain of Kir3.1, we modeled the missing segment using the loop modeling interface of the Rapper server (http://raven.bioc.cam.ac.uk/loop2.php). The corresponding residues that were modeled were 58–67. In order to connect the C-terminus in the crystallographic structure of Kir3.1 with KirBac3.1, the segment from 181–188 was modeled using conformational memories using an extended conformation as the starting coordinates of the segment. The method of conformational memories.$^{41,42}$ Included two phases: the exploratory phase and the biased sampling phase. Monte Carlo$^{43}$ simulations of the TM2 were performed by varying the torsional angles. Backbone dihedral
angles were restrained to $\pm 50^\circ$ from the values of dihedral angles in $\alpha$-helices: $(\phi, \psi) = (-57^\circ, -47^\circ)$. Side-chain dihedral angles were rotated freely. In each round in the exploratory phase, repeated runs of Monte Carlo simulated annealing were performed from a starting temperature of $T_i = 4000K$, with a cooling schedule of $T_{n+1} = 0.97T_n$ and 30,000 steps per temperature to reach 295K. In the sampling phase, the starting temperature in each round was $T_i = 897K$. Analysis of the resulting conformations was performed at $T_n = 295K$. The environment was modeled by a distance-dependent dielectric. The CHARMM force field was used for the energy calculation. To reach convergence of the resulting conformations 200 rounds of independent random simulations were performed for each construct. The side-chains of the residues in the KirBac3.1 homology model part were constructed using CHARMM version 26. The orientations of the side-chains of the structure obtained were then minimized using the Steepest Descent and the adopted-basis Newton Raphson algorithms.

RESULTS

Kir3.1/Kir3.4, KCNQ1/KCNE1 and TREK1 channels are differentially regulated by phosphorylation. To first assess the PKA effects on each channel, we applied the forskolin, which activates PKA through direct stimulation of adenyl cyclase, (50 $\mu$M) and the PKA inhibitor H-89 (50 $\mu$M) and measured their effects on the activity of Kir3.1/Kir3.4, KCNQ1/KCNE1 and TREK1 channels expressed in Xenopus oocytes (Fig. 1A and B). Kir3.1/Kir3.4 currents were inhibited by H-89 but not affected by forskolin. KCNQ1/KCNE1 currents were inhibited by H-89 and potentiated by forskolin. TREK1 currents were potentiated by H-89 but not significantly regulated by forskolin. In some batches of oocytes a small but significant inhibition of TREK1 activity by forskolin was observed. These findings are consistent with data published for all three channels in terms of their regulation by PKA. In addition, direct phosphorylation by PKA has been shown biochemically for Kir3.1/Kir3.4 and KCNQ1/KCNE1 channels.

H-89 has been shown to regulate other protein kinases besides PKA. To investigate whether the H-89 effects were specific to PKA, we tested several other kinase inhibitors for kinases known to be inhibited by H-89. We tested the PKG inhibitor KT5823 (10 $\mu$M), the AMP-activated protein kinase inhibitor RBl (20 $\mu$M) (Indirubin 3‘-oxime), the casine kinase 2 inhibitor DRB (100 $\mu$M) (5,6-Dichlo robenzimidazol-1-b-ribofuranoside), the MLCK (myosin light chain kinase) inhibitor ML-9 (10 $\mu$M), the Ca$^{2+}$/calmodulin-dependent protein kinase inhibitor KN-62 (10 $\mu$M) and the Akt inhibitor API2 (2 $\mu$M)). The only significant effects observed by any of the inhibitors in any of the channels were an increase of TREK1 channel activity by KN-62 and DRB (23 $\pm$ 9%, n = 33 and 27 $\pm$ 9%, n = 32 respectively), which were quite small effects when compared to that of H-89 (150 $\pm$ 20%, n = 26 activation in the same batches of oocytes).

To test whether the lack of a forskolin effect on Kir3.1/Kir3.4 currents was due to the channel being fully phosphorylated by PKA prior to the forskolin treatment, we coexpressed the channel with Gi-coupled M2 receptors (Fig. 1C). Kir3.1/Kir3.4 channels are normally expressed together with M2 receptor in cardiac cells and are activated by M2 receptor stimulation through the $\beta_y$ subunits of PTX-sensitive G proteins. PTX-sensitive Gi/o subunits are known to inhibit adenyl cyclase, cAMP and PKA-dependent phosphorylation. Muscarinic acetylcholine receptors are also known to exhibit constitutive activity in the absence of ACh. It has been previously shown that Gi-coupled receptors exhibit constitutive activity in both heterologous expression systems as well as native heart cells to inhibit cAMP synthesis. M2 receptor coexpression decreased Kir3.1/Kir3.4 basal activity and revealed forskolin regulation of channel activity. These results are consistent with the interpretation that Kir3.1/Kir3.4 channels are fully phosphorylated by PKA without, but not with M2 receptor coexpression. In a similar manner, expression of Gs receptors has been shown to regulate basal PKA phosphorylation levels of IKs channels.

In order to directly test whether the C-termini of the Kir3.1 and Kir3.4 subunits could be phosphorylated, we used purified GST-Kir3.1 and GST-Kir3.4 C-termini and incubated them with the catalytic subunit of PKA. Figure 1D shows that both the Kir3.1 and Kir3.4 C termini were phosphorylated by PKA, as indicated by the stronger bands in phospho-protein staining (upper panel). The fluorescent phospho-protein probes bind phospho-serine, phospho-threonine, and phospho-tyrosine with higher affinity than to non-phosphorylated residues. The stronger bands did not result from a greater amount of proteins, as the total protein staining showed similar amounts of proteins in the absence and presence of PKA (lower panel). The PKA phosphorylation did not occur on the GST-tag but rather on the Kir3 subunits, as control experiments with purified GST tag alone showed similarly faint bands in phospho-protein staining in the absence and presence of PKA. Ovalbumin and casine that are constitutively phosphorylated proteins, were used as positive controls to show that the phospho-protein staining probes worked properly. These in vitro results establish that the C termini of the Kir3 subunits can be phosphorylated by PKA.

Kir3.1/Kir3.4, KCNQ1/KCNE1 and TREK1 channels are inhibited by stimulation of M1-receptors, and agonist-induced inhibition is differentially regulated by PKA. Several G-protein coupled receptors are known to couple to PLC through Gq/G11 proteins. Upon receptor stimulation, PLC hydrolyzes PIP$_2$. For several PIP$_2$-activated channels, PIP$_2$ hydrolysis leads to current inhibition. These include Kir3.1/Kir3.4, KCNQ1/KCNE1 and TREK1 channels. To measure PLC-regulation of each of the channels we coexpressed Kir3.1/Kir3.4, KCNQ1/KCNE1 and TREK1 channels with the Gq coupled muscarinic type 1 receptor (M1) receptor in Xenopus oocytes and showed that the corresponding currents were all inhibited by ACh application (Fig. 2A).

Endogenous Ca$^{2+}$-activated Cl$^-$ currents were used to monitor M1 expression. CF currents developed quickly after ACh application, but inactivated rapidly, not contributing significantly to the Kir3.1/Kir3.4 and TREK1 currents measured in the later phases of the experiment. To avoid such contamination by the I$_{CLCa}$ in KCNQ1/KCNE1 experiments, we coexpressed the M1 receptor with the IP$_3$ phosphatase that abolishes IP$_3$-induced Ca$^{2+}$ release and consequently the Cl$^-$ currents (data not shown).

For Kir3.1/Kir3.4 work from our laboratory (see accompanying paper) has shown that PLC-mediated inhibition involves two interdependent components, one being PIP$_2$ depletion and the other PKC modulation of the channel's affinity for PIP$_2$.

In order to study whether PKA regulates Gq-coupled receptor inhibition of these channels, we pretreated oocytes for at least one hour with either H-89 or forskolin and compared the level of ACh inhibition with that of the untreated control (Fig. 2B and C). ACh inhibition of Kir3.1/Kir3.4 currents was more pronounced for the H-89-treated oocytes. For KCNQ1/KCNE1, a slow activation phase followed channel inhibition. Channel inhibition was measured as the minimum channel current 200 s after ACh application, a time point...
PKA modulates K+ channel sensitivity to PIP2.

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at which the activation phase was not significant and it allowed us to assess the effects of PKA on the inhibitory phase. The ACh inhibition of KCNQ1/KCNE1 currents was weaker for forskolin-treated and stronger for H-89-treated oocytes as compared to control. Finally, ACh inhibition of TREK1 currents was less pronounced for H-89 treated oocytes compared to untreated controls. All experiments were performed with constant perfusion of either H-89, forskolin or the solvent DMSO (control) for each one of the groups tested. Our results suggest that PKA regulates M1 inhibition for all three channels.

PKA phosphorylation of the channels is responsible for the regulation of agonist-induced inhibition. To test whether PKA phosphorylation of each of the channels is responsible for the PKA modulation of the observed PLC-dependent inhibition, we mutated consensus phosphorylation sites in each of the three channels.

Medina and colleagues26 have suggested that Kir3.1 was the predominant phosphorylated subunit of native atrial K_ACh channels. We tested whether mutations of putative PKA phosphorylation sites in Kir3.1 could affect the H-89 inhibition observed for Kir3.1/Kir3.4 channels. Mutations in two putative phosphorylation Kir3.1 sites, S221 and S315, abolished the H89 effects on Kir3.1/Kir3.4 currents (Fig. 3A, left panel). Current activity was not significantly different with the S221D/S315D double mutants as compared to the S221A/S315A double mutants (data not shown), suggesting that the negatively charged mutant does not mimic the functional
PKA modulates K+ channel sensitivity to PIP2

PKA modulates K+ channel sensitivity to PIP2. The kinase phosphorylation prediction of these residues is not restricted to PKA, suggesting they might also be phosphorylated by additional kinases in the native tissue. All Kir3.1 mutant subunits tested show a lower activity upon coexpression with wild-type Kir3.4 when compared to the wild-type subunit (10–20% of wild-type current). The average wild-type current was 15.3 ± 1.1 μA measured at -80 mV (n = 31). Both the Kir3.1 serines probed are predicted to be phosphorylated (see Methods).52

We also measured the PKA modulation of M1-dependent inhibition for the PKA-insensitive mutants. Each of the mutations of S221 and S315 to either Ala (shown) or Asp (not shown) abolished the PKA modulation of M1 inhibition (Fig. 3B, left panel). This result is consistent with the interpretation that direct channel phosphorylation by PKA is responsible for the PLC-dependent modulation observed.

KCNQ1/KCNE1 channels have been shown to be directly phosphorylated by PKA.29 The KCNQ1 subunit was shown to be phosphorylated in the S27 position (Fig. 3A, middle panel). We found that in the Xenopus oocyte expression system mutating just S27 to either an Asp (shown) or an Ala (not shown) did not completely abolish the effects of PKA phosphorylation on normalized current. It has been shown that in oocytes the N-terminus of the KCNQ1 subunit is responsible for the phosphorylation of the channel. Thus, we mutated a second putative PKA phosphorylation site S92 (Fig. 3A, middle panel). Mutation of S92 alone did not abolish the PKA regulation of the channel (Fig. 3A, middle panel). The double mutant S27D/S92D, however, completely abolished the effects of PKA regulation on current level (Fig. 3A, middle panel). S92 is a residue predicted to be phosphorylated in KCNQ1 (see Methods).52 Our results do not exclude phosphorylation of the S92 residue by

Figure 2. PKA modulates the M1 muscarinic receptor inhibition of Kir3.1/Kir3.4, KCNQ1 and TREK1 currents. (A) Channels were coexpressed with M1 receptors: (left) representative trace of Kir3.1/Kir3.4 whole-cell currents in a ramp protocol (200 mV/s) before and after 10 μM ACh application; (center) representative trace of KCNQ1/KCNE1 whole-cell currents depolarized to +40 mV from a -80 mV holding potential before and after 10 μM ACh application; (right) representative trace of TREK1 whole-cell currents in ramp protocol before and after 10 μM ACh application. (B) H-89 and forskolin were perfused during ACh application. Representative trace of the effect of ACh on whole-cell currents with and without H-89 treatment. Dotted line shows zero current level. (Left) Kir3.1/Kir3.4 currents measured at -100 mV in 96 mM K+ extracellular solution; (center) KCNQ1/KCNE1 currents measured at +40 mV after a 2 s depolarizing pulse from -80 mV; (right) TREK1 currents measured at + 80 mV. Solutions as in Figure 1. Currents were normalized to maximal whole-cell current. (C) Summary data for current inhibition by ACh (for experiments performed as in B). Kir3.1/Kir3.4 currents (n = 12–13), KCNQ1/KCNE1 currents (n = 9–12) and TREK1 currents (n = 17–21). Zero current level was estimated by either 5 mM Ba2+ application [Kir3.1/Kir3.4] or leak subtraction. Leak was calculated for KCNQ1/KCNE1 channels and TREK1 channels by measuring the current at -80 mV in low K+ solutions.
PKA modulates K⁺ channel sensitivity to PIP₂.

**Figure 3.** Mutations in putative PKA phosphorylation sites abolish PKA regulation of M1 inhibition. (A) Summary currents for Kir3.1/Kir3.4 mutant channels (n = 5–13), KCNQ1/KCNE1 mutant channels (n = 7–28) and TREK1 mutant channels (n = 23–24) were measured as in Figure 2 for wild-type channels and each of the mutants indicated. Currents were normalized to the average untreated current for Kir3.1/Kir3.4 and KCNQ1/KCNE1 channels. For TREK1 mutants currents were normalized to the average wild-type untreated current. For Kir3.1 and TREK1 mutants each of the mutants abolished PKA regulation of channel activity. For KCNQ1/KCNE1(Q1/E1) both the single S27D and S92D mutations decreased but did not completely abolish channel regulation by PKA. The double mutant S27D/S92D coexpressed with KCNE1 was insensitive to both H-89 and forskolin treatment. (B) Summary data for current inhibition by ACh (for experiments performed as in A).

other kinases besides PKA in native cells. The KCNQ1 mutants show an activity level similar to the wild-type channel. Average wild-type current was 6.7 ± 0.5 μA measured at +40 mV after a 2 s depolarization (n = 61).

We next measured the PKA modulation of PLC-dependent inhibition of KCNQ1/KCNE1 channels for the mutants that affected PKA regulation of the channel. Mutations of the S92 (Fig. 3B, middle panel) but not of the S27 residue (not shown) completely abolished the PKA regulation of M1 inhibition of KCNQ1/KCNE1 currents, suggesting that the phosphorylation of this residue is critical for the crosstalk observed. The double mutation of the S92 and S27 residue also completely abolished the PKA regulation of M1 inhibition of KCNQ1/KCNE1 currents (data not shown).

TREK1 channels have been functionally shown to be directly phosphorylated by PKA at the S333 position. We mutated this site to either an Ala or an Asp and either mutation abolished the functional effect of H-89 consistent with the published data (Fig. 3A, right panel). TREK1(S333A) displayed larger currents than the TREK1(S333D) mutant (Fig. 3A, right panel). Average TREK1 wild-type current was 13.0 ± 0.7 μA measured at +40 mV (n = 61).

We measured PKA modulation of PLC dependent TREK1 regulation for the PKA-insensitive mutants. Either mutation abolished PKA modulation of the PLC-dependent inhibition. Mutant channel currents in cells treated with H-89 were not significantly different than mutant channel currents in untreated cells (Fig. 3A, right panel). These data suggest that phosphorylation of the S333 residue is necessary for regulation by M1 stimulation (Fig. 3B, right panel). The mutant channels expressed in cells treated with H-89 exhibited a small but significantly larger inhibition by M1 than untreated cells. There could be a small regulatory effect by a different site on TREK1 by PKA that is now revealed by the mutation of the main regulatory site of TREK1.

Taken together, these results show that mutations on putative phosphorylation sites of all three channels abolished both the functional effects of PKA levels on current level and the regulation by M1-mediated inhibition, suggesting that direct phosphorylation of all three channels modulates M1-mediated inhibition.

**PKA regulation of Wortmannin inhibition correlates with PKA regulation of agonist-induced inhibition.** Wortmannin at low nanomolar concentrations blocks specifically PI3 kinases but at micromolar concentrations it also blocks the activity of specific PI4 kinases. Since PI4 kinases are required for the synthesis of PIP₂, inhibition of specific PI4 kinase enzymes can interfere with replenishment of PIP₂, thus decreasing the fast turnover of membrane PIP₂ levels. Wortmannin inhibition has been shown to correlate with channel sensitivity to PIP₂. More sensitive channels show lower inhibition than less sensitive ones.
PKA modulates K+ channel sensitivity to PIP$_2$.

To test whether PKA affected sensitivity of the channels to PIP$_2$, we measured the PKA sensitivity to wortmannin treatment. Oocytes expressing Kir3.1/Kir3.4, KCNQ1/KCNE1 or TREK1 channels were incubated with wortmannin and effects on three groups were compared: untreated (control) and treated either with H-89 or forskolin. Concentrations of wortmannin that block about 50% of channel activity were used for each of the three channels: 5 µM for Kir3.1/Kir3.4, 1.25 µM for KCNQ1/KCNE1 and 2.5 µM for TREK1 channels. Cells treated for 60–90 min exhibited suppressed channel activity. Wortmannin showed a smaller inhibitory effect on Kir3.1/Kir3.4 channels expressed in untreated oocytes than in oocytes treated with H-89 (Fig. 4, left panel). Wortmannin showed in order of increasing effects the smallest inhibitory effect on KCNQ1/KCNE1 channels treated with forskolin, followed by untreated oocytes and then oocytes treated with H-89 (Fig. 4, middle panel). On the other hand, wortmannin showed in order of decreasing effects the largest inhibitory effect on TREK1 channels expressed in oocytes treated with forskolin, followed by untreated oocytes and then oocytes treated with H-89 (Fig. 4, right panel). PKA modulation of wortmannin inhibition on all three channels correlated well with the PKA modulation of M1 inhibition of similarly treated channels, suggesting that the observed effects are related and that the sensitivity of the channels to PIP$_2$ is being modulated by PKA.

Stimulation of β1-Adrenergic receptor regulates agonist-induced inhibition of IKs. Because the KCNQ1/KCNE1 channel is well known to be activated by β1 adrenergic receptor stimulation, we tested whether PKA phosphorylation through stimulation of β1-
PKA modulates K\(^+\) channel sensitivity to PIP\(_{2}\). 

**Figure 6.** PKA regulates K\(^+\) channel inhibition by strengthening channel-PIP\(_{2}\) interactions. (A) left panel. Representative traces of normalized current traces from HEK-293 cells expressing M1, M2 and Kir3.1/Kir3.4 channels; control (n = 8), H-89 (n = 7) and forskolin (n = 8). Treatment with either H-89 or forskolin is marked. Right panel. Summary data for % inhibition of the currents after 30 s ACh application for cells treated as in the left panel. (B) Normalized dose response for single-channel recordings from oocyte membrane patches expressing Kir3.1/3.4. Left panel, Representative trace of channel activities recorded at -100 mV. Middle panel: Dose-response curves were fitted with the Hill equation:

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y = \frac{A_1 - A_0}{1 + \left(\frac{x}{EC_{50}}\right)^h} + A_0
\]

where, A0 = offset, A1 = full amplitude, x = PIP\(_{2}\) concentration, h = Hill coefficient. Right panel: right panel shows an EC\(_{50}\) summary bar plot from the control group (H-89 pre-treated group) and the PKA-treated group. EC\(_{50}\) = 45.4 ± 7.5 μM (n = 5) for the control group and 13.0 ± 2.9 μM for the PKA-treated group (n = 8) (p < 0.001). Kir3.4/3.4, 3+3 ng/oocyte injected 24–72 hours. Oocytes were incubated in OR2 with 50 μM H89 for 1–2 hours before recordings. PKA catalytic subunit 2,500 units/ml was applied with 2 mM ATP-γs.

The β\(_1\) adrenergic receptor also modulated PLC-mediated inhibition for the KCNQ1/KCNE1 channel. We expressed the human KCNQ1/E1 channel in *Xenopus* oocytes. We coexpressed along with the channel subunits the β\(_1\)-adrenergic and M1 receptors and measured M1 inhibition of these channels before and after stimulation of the β\(_1\) receptor with isoproterenol (ISO). The β\(_1\)-adrenergic receptor couples to Gs proteins to stimulate adenylyl cyclase and increase cAMP levels and PKA activity. M1-mediated inhibition was significantly diminished in the background of ISO stimulation (Fig. 5A). The effect was similar to the decrease in M1 inhibition seen in cells treated with the PKA activator forskolin (Fig. 2C, middle panel). The KCNQ1(S92D)/E1 mutant channel did not exhibit β\(_1\) regulation of M1 inhibition (Fig. 5B) suggesting that direct phosphorylation of the channel mediated the β\(_1\) regulation of M1 inhibition.

We performed similar experiments with native cardiac KCNQ1/1 currents (IKs), measuring phenylephrine (Phe) effects before and after isoproterenol (ISO) stimulation. Phe, which is a β\(_1\) adrenergic receptor agonist that stimulates Gq proteins, inhibited IKs, and this inhibition was significantly reduced after cell treatment with ISO (100 nM) for 2 min (Fig. 5C).

These results suggest that physiological modulation of PKA phosphorylation by stimulation of β\(_1\) adrenergic receptors modulates PLC-dependent regulation of the IKs channel both in native and in heterologous systems. In addition, phosphorylation of the S92 site seems to be crucial to this PKA modulation of PLC-dependent inhibition.

**PKA regulates Kir3.1/Kir3.4 delayed inhibition in mammalian cells.** Kir3.1/Kir3.4 channels are normally expressed together with the M2 receptor in cardiac cells and are activated by M2 receptor stimulation through the β\(_3\) subunits of PTX-sensitive G proteins.\(^8\)

To test the consequences of PKA phosphorylation in a mammalian cell system, we measured PKA modulation of PLC-dependent regulation of Kir3.1/Kir3.4 channels after M2 stimulation. We coexpressed in HEK-293 cells Kir3.1/Kir3.4, M1 and M2 receptors. ACh stimulation of the M2 receptor activates Kir3 currents. We measured the effect of forskolin and H-89 treatments on the degree of inhibition shown for Kir3.1/Kir3.4 currents when ACh was applied stimulating both M2 and M1 receptors. H-89 treatment accelerated the kinetics of inhibition (Fig. 6A). Depletion of PIP\(_{2}\) from the channel by stimulation of the Gq/11-coupled M1 or M3 receptors has been shown to underlie inhibition of this channel in both native and heterologous expression systems.\(^8\) Our results indicate that PKA modulates the PLC-dependent inhibition of both basal and agonist-induced Kir3.1/Kir3.4 currents.
PKA regulation shifts the PIP$_2$ dose response of Kir3.1/Kir3.4 channels. In order to directly test whether PKA modulation alters the sensitivity of the channel to PIP$_2$, we measured the dose-response to diC$_8$-PIP$_2$ in patches expressing several Kir3.1/Kir3.4 channels before and after treatment with the PKA-catalytic subunit. Figure 6B (left panel) shows a representative experiment. In order to suppress the channel protein phosphorylation before PKA treatment, oocytes were incubated in H-89 for 1–2 hours before the experiment. Applications of diC$_8$-PIP$_2$ increased the open probabilities (nPo) for this channel. Patches pretreated with PKA-catalytic subunit were significantly more sensitive to diC$_8$-PIP$_2$ activation. The values for PIP$_2$, EC$_{50}$ were 45 ± 7.5 μM for the control group (n = 5) and 13.0 ± 2.9 μM for the PKA-treated group (n = 8) (p < 0.001). These results provided direct evidence that PKA phosphorylation increases the apparent affinity of the channel for PIP$_2$.

**DISCUSSION**

Despite decades of intensive research, much remains to be learned regarding the mechanism of the regulation of ion channel activity through signaling mechanisms that utilize PKA-mediated phosphorylation or hydrolysis of PIP$_2$. PIP$_2$ regulation of membrane transport proteins is an emerging theme in signal transduction. Here we show that channel regulation by PIP$_2$ hydrolysis and PKA-dependent phosphorylation crosstalk. PIP$_2$ regulation of three K$^+$ channels representing each of the major K$^+$ channel families, can be modulated directly through specific channel phosphorylation sites. Our data suggest a general mechanism of regulation of cell excitability: regulation of channel-PIP$_2$ interactions by direct channel phosphorylation.

Most ion channels are physiologically regulated by phosphorylation. Neurotransmitter and hormone stimulation, regulate different signaling pathways that adjust the protein phosphorylation status for particular physiological needs. In addition, drugs or disease pathologies can also alter the cell phosphorylation state. Cholinergic and beta-adrenergic stimulations of ion currents are major physiological mechanisms in the regulation of heart rate and contractility. Stimulation of B1-adrenergic receptors is known to regulate among others, delayed rectifier channels, K$_{ATP}$ channels, Ca$^{2+}$ channels, Na$^+$ channels. Although the direct regulation of all these channels by phosphorylation has been the subject of intense study, the details of how such regulation is affected through crosstalk with different signaling pathways are not well understood. Here we study the crosstalk of these regulatory pathways with another very important signaling pathway, the Gq-coupled receptor signaling. Stimulation of Gq-coupled receptors lead to depletion of membrane PIP$_2$. An increasing number of ion channels and transporters have been shown to be regulated directly by PIP$_2$. Thus, we tested the effects of the PKA phosphorylation and Gq-mediated hydrolysis of PIP$_2$ in three channels that have been shown to be modulated by PKA-dependent phosphorylation and also require the presence of PIP$_2$ for maintenance of their activity. Our data are compatible with a model in which phosphorylation modulates channel activity by regulating channel sensitivity to PIP$_2$ (Fig. 7A). This model is probably a simplification of the complex behavior of these channels, as altering PIP$_2$ interactions might also affect sensitivity to PKA. Phosphorylation and PIP$_2$ probably regulate the channel in a complex manner, each affecting the sensitivity to the other (example in ref. 67). Regardless of the complexity, our data show that PIP$_2$ sensitivity is directly regulated by phosphorylation in representative members from all major K$^+$ channel families.

Inwardly rectifying K$^+$ channels have been characterized best in their interactions with PIP$_2$. The highly conserved N and C terminal cytoplasmic portions of these channels presumably form a binding site that is formed by positively charged residues. Mutations in the PIP$_2$ interacting positive residues of these channels confer lower PIP$_2$ affinity, resulting in either non-functional channels, or channels with lower activity and higher sensitivity to inhibition by agonist-induced PIP$_2$ hydrolysis. Regulation of Kir channel activity by PKA has been long appreciated but the mechanism by which phosphorylation events translate to regulation of activity remain poorly understood. Specifically, regulation of Kir3.1/Kir3.4 channel activity by PKA-mediated phosphorylation has been recognized for more than 15 years and it has been appreciated that the Kir3.1 channel subunit is directly phosphorylated both in vitro and in vivo by kinases, including PKA. Moreover, kinases, phosphatases and A-kinase anchoring proteins have been recognized to form a complex with Kir3.1/Kir3.4 heteromers. We found two putative phosphorylation sites that abolish both the PKA effects and their M1 regulation. Kir3.1/Kir3.4 channels form PKA phosphorylation motifs that are fully conserved in all Kir family members with the exception of Kir5.1 (for the first site), a channel that does not express as a homomer (Fig. 7B). Moreover, the Arg residues that are part of the PKA consensus phosphorylation sites (R-X-S) are fully conserved in all 15 members of the Kir family and have been shown to affect channel-PIP$_2$ interactions for both Kir2.1 and Kir1.1 channels. Figure 7C shows a three-dimensional model of Kir3.1 (see Methods). We have previously identified by site-directed mutagenesis conserved basic residues in both Kir1.1 and Kir2.1 that influence channel sensitivity to PIP$_2$. When we map the corresponding Kir3.1 residues onto the model structure shown in Figure 7C, we see that the majority of the basic residues lie in the juxtamembrane region in a cleft created by the N- and C-termini of each channel subunit. Given this localization of critical basic residues in channel-PIP$_2$ interactions, it is tempting to propose that PIP$_2$ serves as the glue that tethers the N- and C-termini together onto the inner leaflet of the plasma membrane. This conformational state may exert enough mechanical force on proximal gate(s) to cause channel opening. Hydrolysis of PIP$_2$ would exert the opposite effect causing dissociation of the N- and C-termini from the membrane and from one another, which would lead to a collapse of ion channel gate(s) and channel closure. In this scheme, our hypothesis is that phosphorylation of the serine residues allosterically modifies Kir3.1/Kir3.4 interactions with PIP$_2$. This was shown to be the case for several disease related mutations in Kir2.1 and Kir1.1 that are in close proximity to PIP$_2$ binding sites. This effect is unlikely to be achieved by electrostatic interactions, because the presence of the phosphate for the phosphorylated channel or a negative charged amino-acid did not decrease channel-PIP$_2$ sensitivity. It is possible that phosphorylation of S221 and S315 might allow accessibility of R219 and R313 respectively to interact with PIP$_2$, increasing channel-PIP$_2$ sensitivity. It is tempting to hypothesize that this mechanism will be conserved for other Kir family members. Indeed, Kir1.1 regulation by PKA has been suggested to be dependent on PIP$_2$. In our hands the PKA regulation of Kir1.1 or the PKA regulation of Kir1.1 sensitivity to PIP$_2$, with or without expression of the A-kinase anchoring protein, AKAP79, could not be reproduced in the Xenopus oocyte expression system. As a result we could not study whether the mechanism of regulation by direct channel phosphorylation was the same as in the Kir3.1/Kir3.4 channels. Recent studies have also suggested that PKC effects depend on channel-PIP$_2$ interactions for several Kir
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Figure 7. Model of regulation of Kir channel-PIP2 interactions by PKA. (A) Scheme showing a model where PKA phosphorylation sites in the channel directly regulate channel interactions with PIP2, thus regulating the Gq-coupled modulation of the channel. (B) Alignment of the first members of the seven Kir subfamilies showing conservation of the studied PKA phosphorylation motifs. PIP2 interacting sites are marked in blue. In green are residues where naturally occurring mutations associated with disease occur. For the two Ser residues that abolish PKA functional regulation (marked in red): S315 and the putative PKA phosphorylation motif is fully conserved in all 15 Kir family members and S221 is conserved in all but Kir3.1, which does not express as a homomer. (C) Three dimensional structure of two subunits of the Kir3.1 channel (see Methods). PIP2 interacting residues are marked in blue. (right). The two PIP2 interacting residues [R219 and R313] that are part of the PKA consensus phosphorylation sites (blue) and their respective Ser residues (red, S221 and S315) are shown.

PKC phosphorylation has also been suggested to mediate Gq-mediated inhibition for the Kir3.1/Kir3.2 channels, although the sites of PKC action have remained elusive. In summary, our data suggest that modification of channel-PIP2 interactions through direct channel phosphorylation might emerge as a common regulatory mechanism for ion channels (see also ref. 67) with important consequences for neurotransmitter and hormonal regulation of ion channel activity both in normal and disease states.

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