A Switch Mechanism for Gβγ Activation of I_{KAC}*

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G protein-gated inwardly rectifying potassium (GIRK) channels are a family of K⁺-selective ion channels that slow the firing rate of neurons and cardiac myocytes. GIRK channels are directly bound and activated by the G protein Gbg subunit. As heterotetramers, they comprise the GIRK1 and the GIRK2, -3, or -4 subunits. Here we show that GIRK1 but not the GIRK4 subunit is phosphorylated when heterologously expressed. We found also that phosphatase PP2A dephosphorylates a protein in the excised patch abrogates channel activation by Gbg. Experiments with the truncated molecule demonstrated that the GIRK1 C-terminal is critical for both channel phosphorylation and channel regulation by protein phosphorylation, but the critical phosphorylation sites were not located on the C terminus. These data provide evidence for a novel switch mechanism in which protein phosphorylation enables Gβγ gating of the channel complex.

At least eight transmitters activate a class of inward rectifier K⁺ channels via an apparently identical GTP binding protein (G protein)-linked signal transduction mechanism. The G protein-linked receptor subtypes that activate these channels include muscarinic (m2), serotonin (5HT1A), adenosine (P1), somatostatin, enkephalin (µ, κ, δ), α2-adrenergic, and dopamine (D2) receptors (1). G protein-linked receptors couple to a heterotrimERIC protein complex of Ga and Gbg subunits. After these receptors catalyze the transfer of GTP to replace GDP on the Ga subunit, the freed Gbg subunit directly binds and activates the GIRK channel (2–4). These G protein-linked inwardly Rectifying K⁺ (GIRK) channels play a role predominantly in the pacing range of cardiac cells and in the regenerative firing of neuronal cells where they oppose the slow depolarization of such currents as If in heart or Ih in neurons (HCN channel class) and can compensate for inactivation of the M current in neurons. In the classic example, acetylcholine (ACh) secreted from the vagus nerve binds cardiac muscarinic receptors, initiating a sequence of events leading to slowing of heart rate (1, 5, 6).

The GIRK channel class has four members, GIRK1, GIRK2, GIRK3, and GIRK4. GIRK1 is unique among the four in having a long C-terminal tail, whereas GIRK2, -3, and -4 are quite similar. Cardiac I_{KAC} is composed of two homologous inward rectifier K⁺ channel subunits, GIRK1 and GIRK4 (2), which form a heterotetramer consisting of two GIRK1 and two GIRK4 subunits (7). Similar complexes comprised of GIRK1 and GIRK2 (8–10), or GIRK1 and GIRK3 (11, 12) form primarily neuronal G protein-gated K⁺ channels. I_{KAC} channel activity is also modulated by levels of intracellular Na⁺ (13), ATP (2, 13–15), phosphatidylinositol bisphosphate (16, 17), and fatty acids (18). These agents are not required for Gbg activation of the channel. Two mechanisms are plausible for ATP regulation of channel activity. The first mechanism is phosphorylation at one or more sites on the channel or an associated regulatory protein that changes the open probability of the channel. The second proposed mechanism is one in which the level ofPIP₂, a critical cofactor for channel activation, is regulated by ATP (16, 17). Levels of phosphorylation of many proteins are regulated by the coordinated activities of protein kinases and phosphatases. We investigated whether I_{KAC} activity was also regulated by such a mechanism. Here we show that the GIRK1 subunit of I_{KAC} is phosphorylated both in vitro and in vivo. Furthermore, the treatment of inside-out patches with phosphoprotein phosphatase 2A (PP2A) prevents channel activation by Gbg. Site-directed mutagenesis experiments demonstrate that the GIRK1 C terminus is critical for both channel phosphorylation and channel activity regulation by protein phosphorylation.

MATERIALS AND METHODS

Primary Cell Cultures—Atrial auricles from neonatal rat (postnatal day 2) were microdissected from total heart tissue and digested using a myocyte isolation kit according to the manufacturer’s specifications (Worthington, Lakewood, NJ). Dispersed cells were plated onto fibronectin-coated glass coverslips in Dulbecco’s modified Eagle’s medium supplemented with 10% horse serum and 5% fetal bovine serum, and cultured in 10% CO₂ at 37 °C for 2 days.

Cell Culture and Transfection—CHO-K1 and HEK-293 cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal calf serum (Sigma Chemical Co., St. Louis, MO) at 37 °C, 5% CO₂. Cells were plated at 3 × 10⁵ per 100-mm dish or 4 × 10⁶ cells per 35-mm dish, respectively, 1 day prior to transfection. Both cell lines were transfected using TransIT LT-2 (PanVera Corp.). Each 100-mm or 35-mm plate was transfected with 5 and 1.2 μg of plasmid DNA, respectively (40% of each plasmid containing either wild type or mutant GIRK1 or GIRK4 cDNA and 20% of pGREEN Lantern-GFP, Life Technologies, Inc.).

Immunoprecipitation—Bovine atria plasma membranes were isolated as described (2). Membranes were solubilized in immunoprecipitation (IP) buffer containing (in mM): 10 HEPES, 1 EDTA, 1 dithiothreitol, 100 NaCl, and 1.0% Triton X-100 supplemented with protease inhibitors (0.5 mM phenylmethylsulfonfyl fluoride and 2 μg/ml each of leupeptin, aprotinin, and pepstatin). Native I_{KAC} was immunoprecipi-
for 30 min at 37 °C. Lipids were extracted with chloroform/methanol/1
10% dialyzed fetal bovine serum for 2 h followed by 3 h in the same

cells were transferred to the phosphate-free media supplemented with

phosphatase for 20 min at room temperature. Reaction buffers were formu-

lation, the

(m in mM: 140 KCl, 5 EGTA, 10 K-HEPES, 2.0 MgCl₂, pH 7.2) at a final

ated according to the manufacturer’s protocols. For

phosphatases and 32P incorporation visu-

alyzed by autoradiography.

as in A. After washing, immunoprecipitates

ative for 1.5 h at 4 °C with the corresponding antibody and Protein G-Sepharose (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Immunoprecipitates were washed four times with RIPA buffer (15 mM NaCl, 1% Triton X-100, 0.5% sodium deoxy-

cholate, 0.1% SDS, 50 mM Tris-Cl, pH 8.0), followed by two washes with

appropriate phosphorylation buffer. 32P-Labeled cells expressing recombinant GIRK1-AU5/GIRK4 were lysed in IP buffer supplemented with phosphatase inhibitor mixture (in mM): 50 NaF, 1 Na₃VO₄, 5

EDTA, and 0.1 μM okadaic acid. Expressed channel hetero-oligomers or

individual subunits were immunoprecipitated with AU5 (Babco, Rich-

mond, CA), FLAG (Sigma), or anti-CIRN2 antibody.

Phosphorylation—Immunoprecipitated atrial IKₐ,Cₚ was phosphorylated for 30 min at 30 °C in 50 μl of the appropriate kinase buffer containing 10 μM γ-[32P]ATP (5 Ci/mmol), immunoprecipitate from 0.55

mg of the atria membranes, and 1 unit of protein kinase. The reaction

was stopped by boiling in SDS buffer. For the dephosphorylation reac-

tion, the in vitro phosphorylated immunoprecipitate was washed in

phosphatase buffer and incubated with 0.5 unit of appropriate phos-

phatase for 20 min at room temperature. Reaction buffers were formu-

lated according to the manufacturer’s protocols. For in vivo 32P labeling,

cells were transferred to the phosphate-free media supplemented with

10% dialyzed fetal bovine serum for 2 h followed by 0 h in the same

medium containing 1 μCi/ml [32P]orthophosphate (NEN Life Science

Products). Samples were visualized by autoradiography at

Materials—GFP was isolated as described previously (2). Calmodulin

kinase (CaMKI, CaMKII), protein phosphatases (PP1, PP2A, PP2B),

and alkaline phosphatase were purchased from Upstate Biotechnology

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(Lake Placid, NY). Glycogen synthase kinase 3 was purchased from

New England Biolabs (Beverly, MA). Protein kinases A catalytic sub-

unit (PKA), protein kinase C (PKC), and the catalytic subunit of PP2A

were supplied by Promega (Madison, WI). For electrophysiological re-

cordings, both GFP and PP2A were diluted with the recording solution

a few minutes before the experiment by 100- and 10-fold to 50 nM and

0.01 unit/μl, respectively, and kept on ice. PiP₃ (10 μM) was then incubated with protein phosphatase 2A (0.01 unit/μl) or protein phosphatase 2A catalytic subunit (0.02 unit/μl) for 30 min at 37 °C. Lipids were extracted with chloroform/methanol/1

m HCl (1:1:0.1), and PiP₃, PiP, and PI were separated by thin layer chromatography (20). Briefly, lipids were spotted onto a thin layer chromatography plate precoated with 1% potassium oxalate. The sep-

aration of the lipids was performed in 2 μl acetic acid/2-propanol (35/65) overnight, and plates were sprayed with EN/Hance (NEN Life Science

Products). Samples were visualized by autoradiography at

RESULTS

Native Cardiac IKₐ,Cₚ Can Be Phosphorylated in Vitro by a

Variety of Protein Kinases—Cardiac IKₐ,Cₚ immunoprecipitated

with anti-GIRK4 antibody was phosphorylated by the catalytic
Phosphorylation of $I_{KAC\text{H}}$

subunit of PKA and other kinases (Fig. 1). Two phosphorylated proteins bands with electrophoretic mobilities corresponding to the core and glycosylated forms of GIRK1 (2) were revealed after kinase exposure. We had previously shown that the immunopurified $I_{KAC\text{H}}$ channel protein contained only GIRK1 and GIRK4 polypeptides (7). To confirm that the phosphorylated proteins belonged to the $I_{KAC\text{H}}$ complex and were not associated with impurities of unrelated strongly phosphorylated proteins in the aCIRN2 immunoprecipitate, we immunoprecipitated $I_{KAC\text{H}}$ with anti-GIRK1 antibody. The same phosphorylation pattern was obtained for the immunopurified protein, and, in a negative control, neutral rabbit immunoglobulins did not immunoprecipitate the phosphorylated proteins. Incubation of the immunoprecipitated channel with [32P]ATP, but without additional kinases, yielded no phosphorylated proteins and demonstrated that the kinase activity was not strongly associated with the channel. Several protein kinases, including PKA, PKC, and calmodulin kinase (CaMKI and CaMKII), phosphorylated GIRK1 (Fig. 1). Only CaMKII phosphorylated (albeit weakly) a protein with the electrophoretic mobility of the GIRK4 subunit.

To determine candidates for the channel phosphatase, in vitro phosphorylated cardiac $I_{KAC\text{H}}$ was treated with a number of phosphoprotein phosphatases (Fig. 1B). Among the phosphatases tested (PP1, PP2A, PP2B, and alkaline phosphatase), only PP2A effectively dephosphorylated the channel.

**Protein Phosphorylation Is Required for Native $I_{KAC\text{H}}$ Activity**—Many attempts to modify cardiac $I_{KAC\text{H}}$ activity by application of ATP to the intracellular surface of excised patches either simply decreased channel run-down or produced relatively small changes in $I_{KAC\text{H}}$ activity (see Ref. 22 for review). These results indicate that $I_{KAC\text{H}}$ may have already been in a phosphorylated state before these experiments were initiated. If native $I_{KAC\text{H}}$, is phosphorylated under normal recording conditions, then a protein phosphatase rather than ATP (or a kinase) should produce a significant functional effect. Because PP2A was most potent in dephosphorylation of the GIRK1 subunit of $I_{KAC\text{H}}$, we tested the ability of this phosphatase to modify $I_{KAC\text{H}}$ channel activity in inside-out patches. As described previously (e.g. Ref. 23), the application of Gβγ (50 nM) to inside-out patches excised from cultured atrial cardiomyocytes induced a significant increase in the open channel probability ($NP_o$) in this case from 0.01 ± 0.005 to 0.22 ± 0.02 (3 min after Gβγ application; n = 14; Fig. 2, A and C). Channel activity was stable and did not run down during the 10–15 min of recording (n = 5, no inhibitors of phosphatase or ATP were added). The application of PP2A phosphatase (0.01 unit/μl) to an $I_{KAC\text{H}}$, channel-containing patch already activated by Gβγ did not significantly change the channel’s open probability (Fig. 2, A and C) or mean open time (not shown). In contrast, treatment of the patch with phosphatase PP2A prior to application of Gβγ completely prevented channel activation by Gβγ in 18 of 20 experiments (Fig. 2, B and D). Heat-inactivated PP2A did not abrogate activation by Gβγ. In only two experiments under these conditions did Gβγ increase channel activity ($NP_o$), and then only by a relatively small amount (~2-fold). Similar results were obtained using the catalytic subunit of PP2A as well as PP1 phosphatase (5 of 5 experiments). Alkaline phosphatase did not prevent $I_{KAC\text{H}}$ activation.

Patches preincubated with PP2A and Gβγ were effectively activated by Mg-ATP (4 mM) but not by the non-hydrolyzable analog of ATP, AMP-PNP (4 mM; Fig. 2, B and D). The increase of channel activity after exposure to ATP was mainly due to an increase in the frequency of channel opening (50- to 500-fold in different patches; n = 20), because the channel mean open time was not affected significantly (1.04 ± 0.04 and 1.07 ± 0.03 ms, respectively; n = 20). Activation by ATP required Gβγ because application of the ATP before Gβγ did not activate channels in the patch either under control conditions or after pretreatment with PP2A. Activation by ATP was not modified in the presence of protein kinase inhibitors such as staurosporine, KN-62, or lavandustin C.

The Effects of Phosphatase PP2A Are Not Related to PIP$_2$—Phosphatidylinositol bisphosphate (PIP$_2$) appears to be a co-factor in $I_{KAC\text{H}}$ activation (16, 17). ATP might have enhanced channel activity by increasing PIP$_2$ content in the plasma membrane (16, 17). Therefore, one explanation for the effects of phosphoprotein phosphatase PP2A on $I_{KAC\text{H}}$ channel activity could be that PP2A decreased the level of PIP$_2$ and thus inactivated the channel. Although protein phosphatase PP2A is not known to dephosphorylate PIP$_2$, we tested this hypothesis by incubating PIP$_2$ in the presence of PP2A under conditions identical to those in the patch-clamp experiments (see “Materials and Methods”). No hydrolysis of PIP$_2$ by PP2A was detected in these experiments (data not shown).
The effect of PP2A might also be explained by dephosphorylation of one or more membrane-associated proteins, which regulate PIP$_2$ levels in the membrane. However, this suggestion contradicts the results shown in Fig. 2; phosphatase 2A was effective only when added before G$\beta$Y activation of the channel, and it failed to modify activated channels. In contrast, the hydrolysis of PIP$_2$ resulted in the inhibition of the active channel (16, 17). The application of phospholipase C or PIP$_2$ antibodies to IKACh already activated by G$\beta$Y significantly decreased the channel open probability in rat atrial myocytes ($n = 8$ out of 8 patches) as has been previously demonstrated (16, 17). Finally, if PP2A decreased the level of PIP$_2$ in the membrane, then application of a saturating concentration of PIP$_2$ to the PP2A-treated patch should completely restore channel activity, and subsequent application of ATP should not further activate the channel. However, Fig. 3 shows that the application of a saturating concentration of bovine brain PIP$_2$ only partially restored activity of the channel pretreated with PP2A and G$\beta$Y. Subsequent application of ATP induced an additional significant increase in channel activity ($NP_o$) in all experiments ($p = 0.002$, $n = 6$; paired $t$ test). Thus the effect of phosphoprotein phosphatase 2A on GIRK channel activity cannot be explained by PIP$_2$ degradation in the membrane.

Properties of GIRK1/GIRK4 Channels Expressed in Mammalian Cell Lines—To further delineate the molecular mechanism of IKACh inactivation by protein phosphatases, we expressed GIRK1 and GIRK4 subunits in two mammalian cell lines, CHO-K1 and HEK-293 (Fig. 4). In both cell lines expressing the GIRK1/GIRK4 channel, G$\beta$Y induced a robust and highly reproducible activation of the multimeric channel. Channel activity was sustained and did not run down during 10–15 min of recording. As in atrial cardiomyocytes, neither PP2A nor ATP significantly modified channel activity after G$\beta$Y application in HEK-293 or CHO-K1 cells (Fig. 4A). Patch pretreatment with PP2A prior to application of G$\beta$Y completely blocked G$\beta$Y activation in CHO-K1 cells and significantly decreased the extent of channel activation in HEK-293 cells (Fig. 4, B and D). Subsequent ATP application completely restored channel activity in HEK-293 cells but not substantially in CHO-K1 cells (Fig. 4D). Consecutive application of protein kinase A, protein kinase C, glycogen synthase kinase-3, CaMKI, and CaMKII did not significantly enhance the effect of ATP in CHO-K1 cells. Channel inactivation by protein phosphatase 2A suggests that protein phosphorylation by the specific protein kinase could be an important condition of channel activation by G$\beta$Y. The different sensitivity of the channel to ATP in the two expression systems may be the result of a difference in the specific protein kinase activity in CHO-K1 and HEK-293 cells.

In Vivo Phosphorylation of the Expressed Channel—To determine if atrial IKACh was phosphorylated in native cells, we attempted to immunoprecipitate IKACh from primary cultures of neonatal rat atrial cardiomyocytes metabolically labeled with $^{32}$P. Unfortunately, we could not obtain sufficient numbers of cells for detection of IKACh. To circumvent this shortcoming, we expressed the functionally active GIRK1/GIRK4 heteromeric channel in HEK-293 and CHO-K1 cell lines, metabolically labeled the cells with $^{32}$P, and immunoprecipitated the channel. We have previously established that the properties of GIRK1/GIRK4 in HEK-293 cells, CHO-K1, and native cardiomyocytes are functionally indistinguishable (2) and that the mode of channel regulation by PP2A is similar in native cardiomyocytes and expression systems (see previous section). In the steady state, only the GIRK1 subunit of the channel complex was phosphorylated when expressed in HEK-293 cells, as detected by $^{32}$P labeling and immunoprecipitation of the heteromeric GIRK1/GIRK4 channel (Fig. 5). Identical results were obtained for channels expressed in CHO-K1 cells (data not shown). In some experiments, we observed a weakly phosphorylated band with a mobility corresponding to the GIRK4 subunit, but phosphorylation of this protein was not consistently observed. The same result was obtained when the heteromeric channel was immunoprecipitated with anti-GIRK4 antibody. Interestingly, GIRK4 was phosphorylated when expressed alone without GIRK1 (Fig. 5), a condition whose physiological relevance has not been established.
The GIRK1 C Terminus Is Important for Both GIRK1 Phosphorylation and Channel Inactivation by PP2A—Assuming that PP2A inactivation of I_{KCa} is due to GIRK1 serine/threonine residue dephosphorylation, then mutation of the phosphorylated amino acids should produce an inactive channel. GIRK1 has a wealth of putative serine/threonine phosphorylation sites located in its long carboxyl tail region (Fig. 6). Most of these sites are present in the GIRK1 but not the GIRK4 subunit. Based on the results presented so far, we expected that a point mutation at one of these serine/threonine residues would prevent phosphorylation and result in a GIRK1/GIRK4 channel that could not be activated by G_{b}G_{y}. Thus, most of the putative serine/threonine phosphorylation sites on GIRK1 were mutated to alanine (Fig. 6). None of the 18 single point, or 8 paired, amino acid mutations disrupted the ability of G_{b}G_{y} to activate the expressed mutant GIRK1/GIRK4 channel. However, two point mutations, T193A and S221A, resulted in expression of channel with a much shorter mean open time (less than 0.3 ms) and one mutation (S278A) displayed lower sensitivity to G_{b}G_{y}. When these three mutant proteins were expressed in HEK-293 cells, none exhibited decreased levels of GIRK1 phosphorylation. More complex combinations of mutations were also tried. The GIRK1 subunit with a mutation of 3

FIG. 4. PP2A pretreatment modulates the activity of GIRK1/GIRK4 channels expressed in mammalian cell lines. A, 50 nM G_{b}G_{y} increased GIRK1/GIRK4 channel activity in HEK-293 cells, similar to its action in atrial myocytes. Neither PP2A (0.01 unit/μl), nor addition of ATP (4 mM), affected the level of channel activity. B, pretreatment of the patch with PP2A blocked activation by G_{b}G_{y}. C and D, activity of I_{KCa} channel expressed in CHO-K1 and HEK-293 (mean ± S.E.). Channel activity (N_{Po}) was calculated for 0.5- to 2-min stretches of records during steady-state channel activity (usually 1–2 min after drug applications). Asterisks indicate significant differences in D as compared with C (p < 0.05).

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FIG. 5. Only GIRK1 was phosphorylated in vivo in the GIRK1/GIRK4 heteromeric channel. Epitope-tagged GIRK1/GIRK4 subunits were expressed in HEK-293 cells. After in vivo protein labeling with [32P]orthophosphate, channel subunits were immunoprecipitated from the cell lysate with anti-AU5 antibody (mock, GIRK1, and GIRK1 + GIRK4) or anti-CIRN2 (GIRK4) antibody. [32P] incorporation was measured by autoradiography. Expression of GIRK1 and GIRK4 was verified by Western blot with anti-Ceh and -CIRN2 antibody, respectively. The three bands in the GIRK1 lanes represent the core (lower) and two glycosylated forms of GIRK1.

FIG. 6. The putative sites of GIRK1 and GIRK4 phosphorylation. The putative serine/threonine sites of the GIRK1 and GIRK4 subunit phosphorylation were determined using MacVector 6.01 (Oxford Molecular Group PLC). Asterisks indicate the residues on GIRK1 subunit that were mutated to alanine. Vertical arrows mark the GIRK1 truncation sites. The unique region of the GIRK1 C terminus is underlined.
residues (193, 221, and 278) to alanines did not express in either HEK-293 or CHO-K1 cells as determined by Western blot (not shown). The failure of the point mutations to disrupt channel activation by G<sup>βγ</sup> indicated that multiple phosphorylation sites were relevant to GIRK1 function or that non-consensus serine/threonine residues might be involved. Tyrosine phosphorylation of GIRK1 is unlikely to be pertinent, because the phosphorylated and immunoprecipitated channel was not recognized with an anti-phosphotyrosine antibody (data not shown).

Several truncation mutants were constructed to determine the regions of the GIRK1 protein that were phosphorylated in vivo. We found that truncation of GIRK1 after residue 373 resulted in complete disappearance of the phosphorylation signal (Fig. 7). The absence of the phosphorylation was not due to decreased Δ373-GIRK1 subunit expression, because its expression level was even higher than that of the wild type GIRK1 subunit (Fig. 7). Furthermore, the ability of Δ373-GIRK1 to associate with the GIRK4 subunits was not decreased compared with wild type GIRK1 (data not shown, see also Fig. 8 where the functional heterochannel is shown). These results suggested that the phosphorylation site(s) are located on the carboxyl tail of the GIRK1 subunit. To further define the region of the relevant phosphorylation site, we truncated the GIRK1 molecule after amino acid 419. The level of phosphorylation of the Δ419-GIRK1 mutant protein was indistinguishable from that of the wild type subunit (Fig. 7). This suggests that the relevant phosphorylation sites were located between amino acids 373 and 419 of GIRK1. Surprisingly, the simultaneous mutation of all seven serine/threonine residues located between these amino acids did not eliminate GIRK1 subunit phosphorylation (Fig. 7). This suggests that residues between amino acids 373 and 419 are critical for channel phosphorylation, but that the actual phosphorylated sites must be located proximal to amino acid 373.

Membranes isolated from HEK-293 cells and expressing the GIRK1/GIRK4 heterotetramer were treated with PP2A under conditions similar to those in patch-clamp experiments. GIRK1 phosphorylation was not changed under these conditions. But PP2A completely dephosphorylated in vitro phosphorylated I<sub>KACH</sub>, suggesting that the sites phosphorylated in I<sub>KACH</sub> in vitro are different from those phosphorylated in vivo. These results could be explained if PP2A had dephosphorylated a regulatory membrane-associated protein or if it had dephosphorylated residues that were critical for channel activity but that were only a small fraction of the total GIRK1 phosphorylated residues.

We suggested above that expression of a dephosphorylated

FIG. 7. The Δ373-GIRK1 C-terminal truncated mutant is not phosphorylated in vivo. Epitope-tagged full length (WT), Δ373-, and Δ419-C-terminal GIRK1 truncations, or the GIRK1 mutant (7 mut) with its 7 serine/threonine residues replaced by alanine (T377A, S379A, S385A, S396A, T397A, S401A, S407A) were co-expressed in HEK-293 cells with GIRK4 subunits. Phosphorylated proteins were labeled in vivo by 32P, and channel subunits were immunoprecipitated from the cell lysate with anti-AU5 antibody. GIRK1 subunit expression levels were compared on Western blot with anti-KGA-N2 antibodies. The physical association of GIRK4 with truncated ΔC-GIRK1 was confirmed by probing the immunoprecipitates with GIRK4 antibody (data not shown).

![Western blot](image-url)

![32P image](image-url)

FIG. 8. Truncation of the C-tail of GIRK1 prevents channel regulation by PP2A. A, single channel currents from inside-out patches excised from HEK-293 cells expressing GIRK4 and wild type or mutant subunits of GIRK1. Currents were recorded in the presence of phosphatase PP2A (left traces) or G<sub>βγ</sub> applied after phosphatase wash out (right traces). <i>f</i> = 5 kHz. B, mean ion channel activity (quantified as NP<sub>o</sub>) of channels comprised of GIRK4 and mutants of GIRK1 subunits (HEK-293 cells). Experiments were performed as illustrated in Figs. 4A and 4C. Asterisks show values that were significantly different from those recorded from the wild type channel. NP<sub>o</sub> was calculated for 0.5–2 min during steady-state channel activity (usually 1–2 min after drug application). seven mutants, GIRK1 incorporating all of the following mutations: T377A, S379A, S385A, S396A, T397A, S401A, S407A.
GIRK1 subunit should yield an inactive channel. Surprisingly, co-expression of Δ373-GIRK1 with GIRK4 subunits produced single channel currents that had the identical conductance and channel kinetics of those expressed by wild type GIRK1 and GIRK4 subunits (Fig. 8A, see also Ref. 21). However, unlike the wild type channel, Δ373-GIRK1/GIRK4 was not sensitive to PP2A pretreatment (Fig. 8). The removal of the more distal portion (419–501) of the GIRK1 C terminus did not affect channel phosphorylation but (like the Δ373-GIRK1 channel mutant) was not sensitive to PP2A pretreatment (Fig. 8A). The mutation of all GIRK1 serine and threonine residues between amino acids 373 and 419, and co-expressed with GIRK4, yielded a channel that was sensitive to the phosphatase and biophysically indistinguishable from the wild type channel (Fig. 8B). Taken together, these results support the conclusion that the GIRK1 C terminus is critical not only for GIRK1 phosphorylation but that it also mediates the inhibitory effect of the protein phosphatase.

**DISCUSSION**

This study provides direct evidence that both the native IK_{ACh} and expressed GIRK1/GIRK4 channel are phosphorylated. Only the GIRK1 subunit of the heteromeric channel is phosphorylated when co-expressed with GIRK4. We have also shown that protein phosphorylation is an important switch mechanism for IK_{ACh} channel activation; treatment of the inside-out patch with protein phosphatases completely prevented the channel activation by G_{bg}βγ. Finally, we have shown also that the GIRK1 C terminus is critical for both GIRK1 phosphorylation and GIRK1/GIRK4 heteromeric channel regulation by the phosphatase. This discussion addresses the possible mechanisms involved in phosphorylation-dependent regulation of IK_{ACh} activity.

**Protein versus Lipid Phosphorylation**—We have shown that the GIRK1 subunit of the IK_{ACh} channel protein is phosphorylated and that protein phosphatase prevents IK_{ACh} gating by G_{bg}βγ. Several previous papers have shown that ATP enhanced the activity of IK_{ACh} and suggested that phosphorylation was a potential mechanism of channel modulation (13–15, 24). One potential phosphorylation mechanism suggested by Mullner et al. (24) is through β-adrenergic receptor stimulation of PKA-dependent protein phosphorylation. Another hypothesis suggests that IK_{ACh} could be activated through phosphorylation of phosphoinositides (16, 17). Because PIP phosphorylation is a powerful mechanism of IK_{ACh} regulation, we have extensively tested whether the effect of protein phosphorylation on channel activity could involve PIP_{2} hydrolysis and whether the ATP-induced recovery of the channel activity involved additional PIP_{2} phosphorylation mechanisms. Phosphatase 2A is known to be a protein phosphatase, and we demonstrated that it did not directly dephosphorylate the lipid, PIP_{2}. It is possible that PP2A dephosphorylated and regulated the activity of an unknown protein involved in PIP_{2} metabolism that resulted in a decrease in PIP_{2} concentration in the membrane. In that case, however, PP2A should have inactivated the channel, independent of whether G_{bg}βγ was present or not, because PIP_{2} degradation did not depend on G_{bg}βγ (16, 17). The fact that prior activation of the channel by G_{bg}βγ completely blocked channel inactivation by PP2A rules out this possibility. Finally, ATP increases channel activity even in the presence of saturating PIP_{2} concentrations, strongly suggesting that PP2A-sensitive protein phosphorylation is involved in IK_{ACh} gating independent of PIP_{2}.

**Does PP2A Inactivate IK_{ACh}, via GIRK1 Dephosphorylation?**—Our data point to a potential switch mechanism that would effectively block receptor-dependent activation of IK_{ACh} (IK_{GQ} or GIRK channels) by making the channel insensitive to gating by G_{bg}βγ. But the upstream mechanism is still obscure. Is dephosphorylation of the constitutively phosphorylated GIRK1 responsible for the PP2A effect, or is another protein involved in this mechanism? If we assume that PP2A dephosphorylates GIRK1, then mutations of phosphorylation sites critical for channel activation might be expected to result in expression of a non-activatable channel protein. To identify phosphorylated serine/threonine residues, we performed multiple point mutations/deletions of the GIRK1 subunit. Deletion of the GIRK1 C terminus resulted in expression of non-phosphorylated but functionally normal channel. The fact that PP2A did not dephosphorylate in vivo phosphorylated channel does not support a model in which dephosphorylation of the GIRK1 subunit itself is responsible for channel inactivation. Instead, these results suggest that either PP2A dephosphorylated an associated regulatory membrane protein or that it dephosphorylated only a small fraction of the phosphorylated residues in GIRK1 critical for channel activity. If there is an associating protein, it would be likely to be relatively loosely bound to IK_{ACh}, because in at least one study no proteins were found to be associated with GIRK1/GIRK4 subunits after purification of the native IK_{ACh} complex (7).

The unique C-terminal tail of GIRK1 has prompted speculation over several years about its function. Several hypotheses have been advanced, including the proposal that the tail was the site of G_{bg}βγ activation (25, 26). Subsequently, it was found that G_{bg}βγ could activate homomultimeric GIRK2, -3, or -4 channels (2) and that the GIRK1 C terminus was not required even for GIRK1/GIRKx heteromultimer activation (21). The present studies have shown that phosphatase 2A treatment of the in vivo channel abrogates G_{bg}βγ activation of the channel complex and that the GIRK1 C terminus participates in this switch mechanism. The targets of the phosphatase are not the phosphorylated residues on the GIRK1 C terminus, but the C terminus is required for the action of the phosphatase. The simplest model supported by these results is one in which the GIRK1 C terminus is an inhibitor of the channel. This inhibition is removed by phosphorylation of either GIRK1 or an accessory protein. When phosphorylated, the channel complex is competent for activation by G_{bg}βγ. The channel complex is normally in the phosphorylated state and readily gated by G_{bg}βγ when any one of several neurotransmitter receptors are bound. The next objective of work in this area will be to determine if phosphatases can be physiologically induced to switch off the G_{bg}βγ sensitivity of the channel.

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