Heterologous Facilitation of G Protein-activated K⁺ Channels By β-Adrenergic Stimulation via cAMP-dependent Protein Kinase

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Abstract To investigate possible effects of adrenergic stimulation on G protein-activated inwardly rectifying K⁺ channels (GIRK), acetylcholine (ACh)-evoked K⁺ current, IKACh, was recorded from adult rat atrial cardiomyocytes using the whole cell patch clamp method and a fast perfusion system. The rise time of IKACh was 0.4 ± 0.1 s. When isoproterenol (Iso) was applied simultaneously with ACh, an additional slow component (11.4 ± 3.0 s) appeared, and the amplitude of the elicited IKACh was increased by 22.9 ± 5.4%. Both the slow component of activation and the current increase caused by Iso were abolished by preincubation in 50 μM H89 {2-(p-bromocinnamylamino)ethyl]5-isoquinoinesulfonamide, a potent inhibitor of PKA}. This heterologous facilitation of GIRK current by β-adrenergic stimulation was further studied in Xenopus laevis oocytes coexpressing β₁-adrenergic receptors, m₁ receptors, and GIRK1/GIRK4 subunits. Both Iso and ACh elicited GIRK currents in these oocytes. Furthermore, Iso facilitated ACh currents in a way similar to atrial cells. Cytosolic injection of 30–60 pmol cAMP, but not of Rp-cAMPS (a cAMP analogue that is inhibitory to PKA) mimicked the β₁-adrenergic effect. The possibility that the potentiation of GIRK currents was a result of the phosphorylation of the β₁-adrenergic receptor (β₁AR) by PKA was excluded by using a mutant β₁AR in which the residues for PKA-mediated modulation were mutated. Overexpression of the α subunit of G proteins (Gα), adenylyl cyclase, and subsequent phosphorylation of these channels by cAMP-dependent protein kinase (PKA); Osterrieder et al., 1982; Cachelin et al., 1983; Frohnwieser et al., 1997). Acetylcholine (ACh), released from the vagus nerve, on the other hand, negatively regulates heart rate through inhibitory, pertussis toxin sensitive, G-proteins. Gαq inhibits adenylyl cyclase and subsequently PKA, while direct activation of G-protein-activated inwardly rectifying K⁺ channels (GIRK) is achieved by simultaneous release of the β₁γ subunit dimer of G protein (Gqγ) (see review, Dascal, 1997). Little is known about any possible connection of the sympathetic pathway to membrane hyperpolarizing effectors such as GIRKs. Several reports do describe regulation of GIRKs and/or IKACh by (a) transphosphorylation of GDP via nucleosidophosphate kinase, (b) directly by intracellular nucleotides, and/or (c) by protein phosphorylation/dephosphorylation (reviewed by Dascal, 1997). Kim (1990) found that β-adrenergic stimulation and exposure of inside-out patches derived from rat atrial cells to the catalytic subunit of PKA enhanced IKACh, but in other preparations PKA effects on IKACh were absent (Trautwein et al., 1982; Nargeot et al., 1983; Wang and Lipsius, 1995).
We recently found that GTPγS-activated Gαs attenuates Gβγ-induced activation of GIRK channels expressed in Xenopus oocytes in a membrane-delimited manner (Schreibmayer et al., 1996). This raises the possibility that such inhibition may occur in atrial myocytes when sympathetically stimulated. The current study investigates the effects of β-adrenergic stimulation on GIRK channels in native rat atrial cells and in the Xenopus laevis oocyte expression system. We found that, in atrial myocytes as well as in Xenopus oocytes, the main effect of β-adrenergic stimulation is an enhancement of the ACh-induced GIRK current via PKA-catalyzed phosphorylation. The target for this phosphorylation is still unclear, but it is downstream from the β-adrenergic receptor.

MATERIALS AND METHODS

Electrophysiology

Atrial cells were enzymatically disaggregated from hearts of adult Sprague-Dawley rats as described (Dascal et al., 1993) and stored in the incubator for 1–3 d at 37°C in M199 under 95% O2/5% CO2 until electrophysiological recordings were performed. Cells were then pipetted into a plastic petri dish with a diameter of 35 mm, mounted to a Peltier element thermostated object holder of an inverted microscope (IM35; Carl Zeiss, Inc.). Patch-clamp current recordings were obtained with an Axopatch 1D amplifier (Axon Instruments) using fire-polished pipettes with an open tip resistance of 2–4 MΩ. Perforated recordings were performed with an Axopatch 1D amplifier (Axon Instruments) connected to a 486 compatible PC (Steiner Computers) using the Axotape software (Axon Instruments). Evaluation of current recordings was performed using the Fetchan 6.0. Preincubation of oocytes in H89 was performed by placing the oocytes for 2–3 h in ND96 containing 5 × 10–5 M H89 before experimentation (see Perets et al., 1996). Oocytes were Pertussis toxin (PTX) treated by injecting 100 or 200 ng/oocyte of the A-protomer of the toxin (Alomone Laboratories) 3–16 h before the experiment (Sharon et al., 1997).

Molecular Biology

Plasmid vectors were constructed, grown in bacteria, isolated, and linearized using standard procedures (Sambrook et al., 1989). cRNA was synthesized as described (Dascal and Lotan, 1992). The following plasmid vectors were used as described previously: m1R, β2AR (Fidler-Lim et al., 1995); Gαs (Blumenstein et al., 1999); GIRK1wt, GIRK2wt, GIRK4wt (Silverman et al., 1996); CFTR (Uezono et al., 1993); Gαs and Gαi (Jing et al., 1999). The cDNA of β2AR was obtained from Y. Daaka (Daaka et al., 1997) and subcloned into the pGEMHE vector (Liman et al., 1992). To create GIRK1(C40) and GIRK4(C40), we performed a PCR procedure with forward and reverse primers containing the desired parts of the coding sequence of each cDNA preceded or followed, respectively, by restriction enzyme recognition sequences. The PCR products were digested with the restriction enzymes and ligated into the EcoRI and HindIII sites (GIRK1(C40) and Smal and Xbal sites (GIRK4(C40)) of pGEMHE.

Data Normalization and Statistics

To normalize our data (to eliminate scatter introduced by batch-to-batch variation of protein expression in oocyte preparations, different ratios of basal to acetylcholine induced currents for different GIRK isoform combinations or mutants), all currents were expressed as a percentage of the basal HK-induced current of the GIRK1/GIRK4 heterooligomeric channel of a given experimental day and batch of oocytes. Different groups were compared using the unpaired Student’s test (SigmaPlot 2.0; Jandel Scientific). In some instances, the calculated average value of current increase in percent was tested for a significant difference from zero by assuming a normal distribution (Murray and Spiegel, 1961).

Solutions

The composition of the solutions used was as follows (mM): HP: 136 KCl, 4 NaCl, 2 MgCl2, 10 HEPES, buffered with NaOH to pH 7.4; pipette solution: 120 K+ as aspartate, 20 NaCl, 2 MgCl2, 11 EGTA, 1 CaCl2, 2 ATP, 0.1 GTP, 10 HEPES, buffered with KOH to pH 7.4; ND96: 96 NaCl, 2 KCl, 1 MgCl2, 1 CaCl2, 5 HEPES, buffered with NaOH to pH 7.4; NDE: same as ND96, but contained 2.5 mM pyruvate, 0.1% antibiotics (G-1397, 1000 × stock; Sigma;
leaving the macromolecular composition of the cytosol intact (Fig. 1 B). Application of $10^{-6}$ M isoproterenol alone did not result in any detectable $I_{\text{K}_{\text{ACh}}}$ or increase in inward current ($n = 5$, data not shown). When H89, a specific inhibitor of PKA, was included in the patch pipette, the $\beta$-adrenergic effects, both on magnitude as well as on kinetics of $I_{\text{K}_{\text{ACh}}}$, disappeared (Fig. 1).

To investigate the details of the signal transduction pathway leading to the $\beta$-adrenergic effect on $I_{\text{K}_{\text{ACh}}}$, we used a heterologous expression system, the *Xenopus* laevis oocyte. To assess the ability of heterologously expressed $\beta_{2}$AR to activate PKA in *Xenopus* oocytes, we coexpressed $\beta_{2}$AR together with the CFTR, a Cl$^{2-}$ channel that is a known target for PKA phosphorylation (Anderson et al., 1991; Cheng et al., 1991). Even without coexpression of exogenous G$_{\text{G}_{\beta\gamma}}$, isoproterenol produced a
marked increase in inward current, which was equal in magnitude to CFTR inward currents produced by cAMP injections (Fig. 2, A and B). Furthermore, isoproterenol-induced CFTR currents were prevented and/or blocked by cytosolic injections of Rp-cAMPS (Fig. 2 A; summarized in C), demonstrating that the isoproterenol response was entirely due to PKA stimulation and that Rp-cAMPS injections are indeed effective in blocking endogenous PKA in Xenopus laevis oocytes.

Modulation of GIRK channels by β-adrenergic receptors was studied in Xenopus oocytes heterologously expressing muscarinic m2-receptor, β-adrenergic receptors, and two subunits of G-protein–activated, inwardly rectifying K+ channels (GIRK1 and GIRK4, respectively). Heteromeric GIRK1/GIRK4 channels are believed to constitute the atrial GIRK (see Dascal, 1997), although homomeric GIRK4 channels may also contribute to total I_{ACh} (Corey et al., 1998). Currents were recorded at −80 mV, first in a high-Na+, low-K+ physiological medium (ND96), which was then exchanged to a medium with high external K+, low-Na+ concentration (HK; leading to a basal inward current, termed I_{HK}). When isoproterenol was applied together with acetylcholine, a marked increase of I_{ACh} was observed compared with the application of ACh alone (Fig. 3 A). Application of isoproterenol (Iso) when ACh was already present in the bathing solution led to a marked additional increase of I_{ACh}. In contrast to native rat atrial cells, application of isoproterenol alone during HK led to a small but clearly detectable increase in inward current, termed I_{Iso} (Fig. 3 B). These effects of Iso were absent in oocytes that expressed GIRK and m2R, but were not injected with cRNA encoding β2ARs. It has been reported that G-protein βγ-subunits released from β-adrenergic receptors can directly activate GIRK channels in Xenopus oocytes and atrial cells, provided that exogenous Gβ and/or Gαs are overexpressed (Fidler-Lim et al., 1995; Bender et al., 1998; Sorota et al., 1999). To exclude direct G βγ effects and to distinguish them from effects put forth by the cytosolic second messenger branch, 3’5’-cAMP was directly injected into the oocytes during electrophysiological recording. An enhancement of inward current resulted both in the absence and the presence of acetylcholine (Fig. 3 C and E). The current voltage relation of cAMP-induced currents clearly revealed inward rectification (Fig. 3 F). Furthermore, cAMP effects on I_{HK} were absent when cRNA encoding GIRK channels was not injected into the oocytes. The enhancement of the basal activity of GIRK channels by cAMP injection was not altered by β2AR coexpression [59.6 ± 7.5% (n = 55, see Fig. 3 D) vs. 59.7 ± 7.2% (n = 9, data not shown)]. Injection of Rp-cAMPS, a cAMP analogue and inhibitor of PKA, did not enhance the basal inward currents. This fact indicates that nonspecific effects of cyclic nu-
Figure 3. β2-adrenergic facilitation of heterologously coexpressed GIRK currents and cAMP effects in *Xenopus laevis* oocytes. (A) Original current trace recorded at a membrane holding potential of −80 mV. ND and HK denote changes of the superfusion medium from ND96 to HK, bars denote superfusion with agonists. (B) Same as in A, but Iso was applied alone or during ACh. (C) Same as in A, but 30 pmol cAMP was injected during HK before ACh superfusion. (D) Statistics of the effects of isoproterenol superfusion and/or cytosolic injection of cAMP and Rp-cAMPS on basal current amplitude, expressed as percentage of basal I_{HK}. Calculated average values ± SEM are shown; the number of individual cells is given in parenthesis. (**) Mean value deviates significantly (P < 0.01 and 0.001) from the corresponding control group (i.e., oocytes not expressing heterologous β2AR in the case of Iso effects) or cAMP injection into native oocytes in the case of cAMP effects. (Left to right) Iso-induced current on basal HK (with or without coexpressed β2AR), 30–60 pmol cAMP injection during basal HK, cAMP injection into native oocytes, injection of 30–60 pmol Rp-cAMPS during basal HK. (E) Same as C, but cAMP was injected during ACh. (F) Current-voltage relation of the cAMP-induced currents as measured by a triangular voltage pulse (f = 1 Hz). (G) Current induced by cytosolic cAMP injection on basal current, obtained by subtraction of the basal current before cAMP injection from basal current after cAMP injection. (○) Current induced by cAMP injections on ACh-induced currents. (G) Statistics of the ef-
cicles, unrelated to PKA activation, were absent. To better understand the mechanisms of modulation of GIRK via β2AR and Go, in Xenopus oocytes and to substantiate the finding that PKA might stimulate basal and agonist-induced GIRK currents in the oocytes, we overexpressed Go at different concentrations in addition to GIRK1, GIRK4, and m2R. At low doses of injected cRNA encoding Go, basal and ACh-evoked GIRK currents were enhanced, while at higher doses a suppression of both current components was observed. Interestingly, the increase in both basal and agonist-induced currents was inhibited by preincubation of the oocytes in 5 × 10^{-5} mol/liter H89, while the suppression at higher expression levels of Go was H89 insensitive (Fig. 4). These results suggest that the basal activity of the overexpressed Go is sufficient to activate adenyl cyclase, and thus PKA, and to cause an increase in GIRK activity. At higher doses, the free Go most likely sequesters G_{i}, thus causing a decrease in GIRK activity.

In the intact atrium, stimulation of GIRK occurs via PTX-sensitive G proteins of the G_{i}/ Go family (reviewed by Dascal, 1997). Recently, it has been discovered that β2-adrenergic receptors switch their G-protein selectivity from Go to Go when β2AR is phosphorylated by PKA (Daaka et al., 1997). This raised the possibility that the β-adrenergic stimulation of GIRK could result from a direct activation by PTX-sensitive G protein via the PKA phosphorylated β2AR. Therefore, oocytes expressing β2AR_{wt}, m2R, and GIRK1/GIRK4 channels were pretreated with PTX to block the G_{i}/ Go family proteins. While basal inward currents were reduced by the PTX treatment (indicating some contribution of basal-active inhibitory G proteins to the resting inward current), the current induced by isoproterenol was reduced slightly (Fig. 5). Furthermore, instead of β2AR_{wt}, we expressed a mutant β2-adrenergic receptor in which the PKA-dependent selectivity switch was disrupted (β2AR_{P}F; Daaka et al., 1997). Interestingly, β2-adrenergic receptor stimulation persisted (Fig. 5, A and B). PTX treatment of oocytes expressing the mutant receptor resulted in a decrease of basal, agonist-independent current similar to the one observed with wild-type receptors, but the isoproterenol-evoked current was not affected.

Phosphorylation of seven-helix receptors is an important mechanism for the regulation of receptor activity (Pitcher et al., 1998). To discriminate between PKA effects on the receptor(s) and on the channel, GIRK1/GIRK4 channels were expressed without m2R and β2AR. cAMP injections were performed to test for PKA effects on basal GIRK currents. As evident from Fig. 6, basal activity GIRK channels respond to cAMP injections with the usual increase in activity (39.0 ± 7.5% of basal HK; n = 12; mean value differs statistically significant from zero at the P < 0.001 level).
To investigate whether other isomeric forms of GIRK channels are also modulated by PKA, we expressed different subunit compositions: GIRK1/GIRK5, GIRK1/GIRK2, and the homooligomeric GIRK2 channels. For combinations not containing GIRK5, the endogenous GIRK5 subunit was eliminated by coinjection of a subunit-specific antisense oligonucleotide (Hedin et al., 1996). When only cRNA encoding the GIRK1 isoform (flowing via the GIRK1/GIRK5 channels) was greatly reduced when compared with oocytes injected with the same amount of GIRK1 cRNA, but without the oligonucleotide (Fig. 7 D). Coinjection of the oligonucleotide with GIRK1/GIRK4 cRNAs also reduced $I_{\text{ACH}}$ to some extent, indicating that in the previous experiments GIRK1/GIRK5 heterooligomeric channels still contributed to the acetylcholine response. As can be seen from Fig. 7, heterooligomeric GIRK1/GIRK2, GIRK1/GIRK4, and GIRK1/GIRK5, as well as homooligomeric GIRK2 channels respond to cytosolic cAMP injections, both under basal and under agonist-stimulated conditions. The cAMP effect on the homooligomeric GIRK2 channels was even significantly larger than on GIRK1/GIRK4 channels. The carboxy-terminal part of the GIRK subunit is involved in $G_{\beta\gamma}$ binding (Huang et al., 1997), is important for gating (Luchian et al., 1997), and contains a PKA phosphorylation consensus site (Dascal, 1997). Hence, truncated forms of GIRK1 and GIRK4 were generated in which the last 40 amino acids were deleted. These constructs, however, still contained the $G_{\beta\gamma}$ binding regions. Cytosolic cAMP injections enhanced currents of mutant channels comprised of both GIRK1$^{\text{DC40}}$/GIRK4$^{\text{DC40}}$. The cAMP-induced currents had the following magnitude, when normalized to the basal current of the oocytes expressing GIRK1$^{\text{wt}}$/GIRK4$^{\text{wt}}$: cAMP injections on basal currents: GIRK1$^{\text{DC40}}$/GIRK4$^{\text{DC40}}$, $33.3 \pm 8.7\%$ ($n = 9$), compared with $66.1 \pm 8.0\%$ ($n = 40$) for GIRK1$^{\text{wt}}$/GIRK4$^{\text{wt}}$; cAMP injections during ACh superfusion: GIRK1$^{\text{DC40}}$/GIRK4$^{\text{DC40}}$, $67.2 \pm 28.0\%$ ($n = 6$) compared with $100.1 \pm 22.9\%$ ($n = 13$) for GIRK1$^{\text{wt}}$/GIRK4$^{\text{wt}}$. All cAMP effects deviate significantly from zero at the $P < 0.001$ level. Hence, the distal COOH terminus (last 40 amino acids) of the GIRK

Figure 5. $\beta_2$-adrenergic facilitation of GIRK currents is not produced by coupling to inhibitory G-proteins. (A) Original current traces recorded at $-80$ mV. GIRK currents were induced by superfusion of the oocytes with $1 \mu\text{mol/liter isoproterenol}$. (Top) Current trace from an oocyte expressing $\beta_2\text{AR}^{\text{wt}}$ and treated with PTX. (Bottom) Same as in the top, but the oocyte expressed $\beta_2\text{AR}^{\text{PF}}$ instead of $\beta_2\text{AR}^{\text{wt}}$. (B) Statistics of the effect of PTX treatment and/or $\beta_2\text{AR}$ mutation on GIRK facilitation, expressed as a percentage of basal $I_{\text{HK}}$ of the control (no PTX treatment, $\beta_2\text{AR}^{\text{wt}}$ expressed). (Empty bars) Oocytes expressing $\beta_2\text{AR}^{\text{wt}}$; (hatched bars) oocytes expressing $\beta_2\text{AR}^{\text{PF}}$. (+ and -) Oocytes incubated or not treated with PTX. Calculated average values $\pm$ SEM from three different batches of oocytes (three oocytes/batch) are shown. (* and ***) Mean value deviates significantly ($P < 0.05$ and $0.001$) from the corresponding control group; i.e., oocytes without PTX treatment.
channel is not the structural determinant that mediated the PKA effect.

**DISCUSSION**

Our results clearly show that \( I_{\text{KACH}} \) in native atrial myocytes derived from adult rats is enhanced by β-adrenergic stimulation. This result strongly supports the finding of Kim (1990) that application of isoprorenaline to newborn rat atrial cells increased \( I_{\text{KACH}} \) channel activity in cell-attached membrane patches. In *Xenopus laevis* oocytes, heterologously expressed β-adrenergic receptors are able to activate GIRKs (Fidler-Lim et al., 1995; and this study). In view of the results of the current study, only part of this β-adrenergic activation in the heterologous expression system can be attributed to a...
direct $G_{\beta\gamma}$ effect. Furthermore, in native atrial cells, direct G-protein activation by $\beta_2$ARs has been shown to occur only after heterologous overexpression of $G_{\alpha_i}$ (Sorota et al., 1999), of $G_{\alpha_{11}}$ subunits (Bender et al., 1998), or after antisense oligonucleotide knockout of GIRK2 (Wellner-Kienitz et al., 1999). Kim (1990) suggested that $\beta$-adrenergic activation of $I_{K_{ACh}}$ is produced by $G_{\alpha_i}$-induced PKA phosphorylation, but in other preparations, PKA effects on $I_{K_{ACh}}$ were absent (Trautwein et al., 1982; Nargeot et al., 1983; Wang and Lipsius, 1995). In our preparation, the slow onset kinetics as well as inhibition by H-89 indicate that the isoproterenol-induced increase of $I_{K_{ACh}}$ can be attributed to phosphorylation by PKA after activation of $\beta_2$-adrenergic receptors. Interestingly, isoproterenol was ineffective in the absence of acetylcholine in native atrial cells, suggesting that both $G_{\beta\gamma}$ dimers and PKA phosphorylation are required for GIRK activation.

To study the molecular mechanism of PKA-induced GIRK stimulation in more detail, we employed the *Xenopus laevis* oocyte expression system. First, the CFTR Cl$^-$ channels were expressed as reporters of PKA activity, since PKA activation of these channels is well documented (Anderson et al., 1991; Cheng et al., 1991). Cytosolic injections of cAMP or Sp-cAMPS effectively activated CFTR currents to the same extent as heterologously coexpressed $\beta_2$AR (Fig. 2). This finding clearly shows that the coexpressed $\beta_2$AR, via the oocyte's endogenous $G_{\alpha_i}$, is able to activate PKA to the same extent as direct cytosolic injections of the second messenger. Furthermore, cytosolic injections of Rp-cAMPS (a selective inhibitor of PKA) completely abolished $\beta$-adrenergic stimulation of CFTR, demonstrating that PKA activity is completely blocked under these conditions. Further evidence for PKA regulation of GIRK was derived using this system: intracellular injection of cAMP, but not of Rp-cAMPS, stimulated GIRK currents to the same extent as heterologously coexpressed $\beta_2$AR (Fig. 3). This finding is substantiated by the fact that modest overexpression of $G_{\alpha_i}$ leads to an increase of basal (agonist independent) as well as acetylcholine-activated GIRK current that can be inhibited by H-89, a selective blocker of PKA (Fig. 4). At higher levels of overexpression, however, GIRK activity decreases, most likely by sequestering endogenously available $G_{\beta\gamma}$ subunits (see Fidler-Lim et al., 1995). So we conclude that PKA phosphorylation facilitates GIRK channel opening either by direct phosphorylation of the channel protein or of an auxiliary cofactor that interacts with the channel. Desensitization of $\beta_2$AR has been shown to occur via two distinct protein kinases: $\beta$-adrenergic receptor kinase phosphorylates the $\beta_2$AR, thereby triggering binding of $\beta$-arrestin to the receptor, which, in turn, terminates further catalytic activation of heterotrimeric G-proteins (for review, see Pitcher et al., 1998). On the other hand, PKA phosphorylation of the $\beta_2$AR changes the specificity of the receptor from the stimulatory to the inhibitory (PTX-sensitive) heterotrimeric G-protein (Daaka et al., 1997). Since GIRK channels are activated predominantly via inhibitory, PTX-sensitive G-proteins, PKA might promote $\beta$-adrenergic stimulation of GIRK channels by the latter mechanism. In our experiments, PTX treatment reduced basal GIRK currents (both when wild-type and mutant $\beta_2$AR's were expressed), showing that inhibitory PTX-sensitive G-proteins contribute to basal activity in the *Xenopus laevis* oocyte system. Activation of GIRK currents via both $\beta_2$AR$^W$ and $\beta_2$AR$^P$, however, took place with or without PTX treatment. This fact clearly indicates that activation of GIRK via $\beta_2$AR does not occur primarily via $G_{\alpha_i}/G_{\alpha_o}$. On the other hand, PTX treatment significantly reduced isoproterenol-induced GIRK currents in the case of $\beta_2$AR$^W$ (but not of $\beta_2$AR$^P$), indicating that coupling of $\beta_2$AR$^W$ to $G_{\alpha_i}/G_{\alpha_o}$ also contributes to some extent. Hence, $I_{B_2}$ in *Xenopus laevis* oocytes is comprised of direct G-protein effects (both PTX sensitive and insensitive) in addition to the part contributed by PKA. Chen and Yu (1994) found that 8-bromoadenosine 3',5'-cyclic monophosphate incubation, as well as cytosolic injection of the catalytic subunit of PKA, reduced desensitization of GIRK channels that were expressed in *Xenopus* oocytes. This effect was attributed to PKA effects on the coexpressed $\mu$-opioid receptor. Since cAMP injections into oocytes expressing GIRK1/GIRK4 channels without any coexpressed seven-helix receptors were also effective (Fig. 6), we conclude that PKA did not act via the seven-helix receptors in our case.

A strong PKA phosphorylation consensus site has been reported to exist in the very end of the carboxy terminus of the GIRK1 subunit (Dascal, 1997). Hence, truncated forms of both GIRK1 and GIRK4 were generated, which were still functional but were missing the last 40 amino acids containing this site. Our results clearly demonstrate that mutant channels react to cAMP injections essentially in the same manner as the wild type. These data indicate that PKA might exert its effect either via another, hitherto unidentified, site located on one of the channels subunits, or via an unknown cytosolic or membrane delimited cofactor that serves as the direct target for PKA.

In native atrial cells, a heterooligomeric protein comprising the GIRK1 and GIRK4 subunits produces most of $I_{K_{ACh}}$. From our study, it becomes evident that GIRK1/GIRK4 heterooligomeric channels are subject to PKA stimulation in native atrial cells as well as in the heterologous expression system. In addition, we were able to demonstrate that other GIRK subunit combinations are subject to PKA-induced facilitation: GIRK1/GIRK5, GIRK1/GIRK2, and even homooligomeric GIRK2
channels were found to be regulated by PKA in the same manner. This finding indicates that we are dealing with a phenomenon of broad functional significance for GIRK channel physiology. Stimulatory and inhibitory G-proteins are thought to produce opposing effects on cardiac excitability under normal physiological conditions. In the present study, however, this is not the case. The physiological role of such heterologous facilitation could be understood as follows: in the heart, β-adrenergic stimulation is known to act via voltage-dependent Ca\(^{2+}\) and Na\(^+\) channels, thereby promoting depolarization, excitability, and contraction (Osterrieder et al., 1982; Cachelin et al., 1983; Frohnwieser et al., 1997). In the absence of a simultaneous facilitation of membrane-hyperpolarizing inwardly rectifying K\(^+\) currents, the membrane-depolarizing effect might lead not only to an increase in heart rate and force of contraction, but also to abnormal patterns of conduction of excitation and subsequent arrhythmias. A concomitant enhancement of I\(_{\text{Kach}}\) would stabilize the heartbeat under these conditions. The exact physiological role of this newly discovered mechanism, however, remains to be elucidated in further studies.

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Note added in proof: During the review process of this paper, Medina et al. reported at the 1999 APS conference, “Biologics of Potassium Channels: From Molecules to Disease,” that the GIRK1 subunit itself is phosphorylated in vitro by PKA (Medina, I., G. Krapivinsky, P. Kooor, L. Krapivinsky, and D.E. Clapham. 1999. Channel phosphorylation is required for I\(_{\text{Kach}}\) activation by G\(_{\text{bg}}\). Physiologist. 42:17). Submitted: 25 August 1999 Revised: 6 March 2000 Accepted: 7 March 2000

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