Novel Inhibition of Gβγ-activated Potassium Currents Induced by M₂ Muscarinic Receptors via a Pertussis Toxin-insensitive Pathway*

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G₁ protein-coupled receptors such as the M₂ muscarinic acetylcholine receptor (mACHR) and A₁ adenosine receptor have been shown to activate G protein-activated inwardly rectifying K⁺ channels (GIRKs) via pertussis toxin-sensitive G proteins in atrial myocytes and in many neuronal cells. Here we show that muscarinic M₂ receptors not only activate but also reversibly inhibit these K⁺ currents when stimulated with agonist for up to 2 min. The M₂ mACHR-mediated inhibition of the channel was also observed when the channels were first activated by inclusion of guanosine 5'-O-(thiotriphosphate) in the pipette. Under these conditions the M₂ mACHR-induced inhibition was quasi-irreversible, suggesting a role for G proteins in the inhibitory process. In contrast, when GIRK currents were maximally activated by co-expressing exogenous Gβγ, the extent of acetylcholine (ACh)-induced inhibition was significantly reduced, suggesting competition between the receptor-mediated inhibition and the large pool of available Gβγ subunits. The signaling pathway that led to the ACh-induced inhibition of GIRK channels was unaffected by pertussis toxin pretreatment. Furthermore, the internalization and agonist-induced phosphorylation of M₂ mACHR was not required because a phosphorylation- and internalization-deficient mutant of the M₂ mACHR was as potent as the wild-type counterpart. Pharmacological agents modulating various protein kinases or phosphatidylinositol 3-kinase 3-kinase did not affect the inhibition of GIRK currents. Furthermore, the signaling pathway that mediates GIRK current inhibition was found to be membrane-delimited because bath application of ACh did not inhibit GIRK channel activity in cell-attached patches. Other G protein-coupled receptors including M₁ mACHR and α₁A adrenergic receptors also caused the inhibition, whereas other G protein-coupled receptors including A₁ and A₂ adenosine receptors and α₂A and α₂C adrenergic receptors could not induce the inhibition. The presented results suggest the existence of a novel signaling pathway that can be activated selectively by M₂ and M₁ mACHR but not by adenosine receptors and that involves non-pertussis toxin-sensitive G proteins leading to an inhibition of Gβγ-activated GIRK currents in a membrane-delimited fashion.

An enormous variety of G protein-coupled receptors (GPCRs)¹ allow for a large number of extracellular signals to converge on a relatively small number of heterotrimeric G proteins (1), which in turn cause activation of downstream effectors. When a cell receives multiple signals that are transduced through different GPCRs, the integration of multiple signaling events is complex not only because of the fact that the G proteins have multiple effector systems but also because of “cross-talk” between signaling pathways. Recently, evidence has emerged that cross-talk between GPCRs not only can occur between pathways that utilize different classes of G proteins but also within one G protein family (2). The current study presents a new case of cross-talk between signals that are produced by activation of adenosine and muscarinic receptors that are known to couple to the same class of G proteins.

The initial goal of this study was to investigate the regulation and the desensitization of M₂ mACHR- and A₁ adenosine receptor-activated K⁺ channels that give rise to the current known as IₖACh. IₖACh channels were first identified in the supraventricular tissue of the heart, and their regulation by M₂ mACHRs and other GPCRs, such as the A₁ adenosine or lysoosphingolipid receptors, has been the topic of many different studies over the past two decades (3–5). IₖACh channels were characterized as G protein-activated inwardly rectifying K⁺ channels (GIRKs) that are activated by pertussis toxin (PTX)-sensitive G proteins in a membrane-delimited fashion (3, 6–9). It has become clear that GIRK channels are activated upon binding of the Gβγ subunits to the channel (10–12). GIRK channels are heterotrimers of two homologous subunits. GIRK1 and GIRK4 form the cardiac channel (13, 14), whereas GIRK1 and GIRK2 or GIRK3 form certain neuronal channels (15, 16). The GIRK channels have been functionally expressed in a variety of cells including Xenopus oocytes and mammalian HEK and CHO cells (13, 17–19).

Desensitization of GPCR-activated GIRK currents has been observed in native tissues and in heterologous expression systems. We have utilized the GIRK channels as a readout system to study desensitization of G protein-mediated signals for two reasons. First, electrophysiological recording of GIRK currents with the patch clamp technique allows for real time measurement of G protein-mediated signals in intact cells. Second, the GIRK channels conduct potassium ions, which diffuse very rapidly, and the concentration of K⁺ can be controlled; therefore, even after activation of GIRK channels for a long period of time, depletion of intra- or extracellular K⁺ does not occur, and

¹ The abbreviations used are: GPCR, G protein-coupled receptor; mACHR, muscarinic acetylcholine receptor; GIRK, G protein-activated inwardly rectifying K⁺ channel; PTX, pertussis toxin; CHO, Chinese hamster ovary; ACh, acetylcholine; PhE, phenylephrine; AR, adrenergic receptor; GTPγS, guanosine 5'-O-(thiotriphosphate); GABA₉, γ-aminobutyric acid, type B; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; IₖACh, atrial muscarinic K⁺ current; HEK, human embryonic kidney.

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hence desensitization at the level of the channels does not play a major role in desensitization events. Desensitization of GPCR-generated signals can be homologous, where only the stimulated GPCR desensitizes, or heterologous, where the signaling by heterologous GPCRs is inhibited. To distinguish between homologous and heterologous desensitization processes, two different G$_i$-coupled receptors (A$_1$ adenosine and M$_2$ mAChR) were co-transfected into HEK293 cells with GIRK1 and GIRK4. Before and after an initial treatment of one of these receptors with agonist, the response to stimulation of the other receptor was determined. If the response of the treated receptor only was reduced after the initial treatment with agonist, this was considered homologous desensitization. However, if there was a reduction of the response of the untreated receptor after an initial treatment of the other receptor, this was considered to reflect heterologous desensitization. We report novel observations concerning heterologous desensitization of GPCR-activated GIRK channels.

MATERIALS AND METHODS

Cell Culture and Transfection—Chinese hamster ovary (CHO-K1) cells, human A1 or A3 adenosine receptors (in CLDN 10B, 0.2 g; a gift from Dr. H. A. Main, Germany) for voltage control, data acquisition, and data evaluation. I$_{K,AC}$ was measured as an inward current using a holding potential of ~90 mV as described (24). Voltage ramps (from $-120$ mV to +60 mV in 500 ms, every 10 s) were used to determine current-voltage relationships.

RESULTS

Ado Induced an Inhibition of Ado-evoked GIRK Currents—M$_2$ mAChRs, as well as A$_1$ adenosine receptors, can activate inwardly rectifying potassium currents carried by GIRK channels when the receptors and channel subunits are transiently co-expressed in CHO K1 cells. Agonist-activated currents were measured, using the whole cell-patch clamp configuration, as inward currents by setting the membrane potential negative (~90 mV) to the potassium equilibrium potential of about ~50 mV (20 mV external potassium). In cells transfected with the GIRK subunits and the M$_2$ mAChRs, application of Ado (1 $\mu$m) caused an activation of GIRK currents that rapidly desensitized during a 2-min application of Ado (Fig. 1A, left panel). In contrast, in cells transfected with the GIRK subunits and the A$_1$ adenosine receptors, rapid superfusion of the cells with adenosine (1 $\mu$m) induced an inwardly rectifying K$^+$ current that exhibited only a marginal desensitization after a 2-min application of adenosine, and after washout of the agonist, GIRK currents deactivated to basal levels (Fig. 1A, right panel).

To determine whether the desensitization of the ACh response took place at the level of the M$_2$ mAChR or downstream of it, we analyzed the effects of a series of successive exposures to Ado and ACh in cells co-expressing both the A$_1$ adenosine receptors and the M$_2$ mAChRs (Fig. 1B). An initial exposure to adenosine induced GIRK currents that were stable after an initial rapid, small desensitization (this type of desensitization is not the topic of this study but was studied in recent papers (25–27)). After washout of Ado and a return of currents to basal levels, the cells were exposed to ACh. The ACh-induced currents were of similar amplitude compared with those induced by Ado; however, again the ACh-induced GIRK currents markedly desensitized during the 2-min exposure to ACh. To test if the ACh treatment would affect subsequent effects of Ado, we exposed the cells to a second application of ACh within 1 min after the washout of ACh (Fig. 1B). These Ado-elicited GIRK currents were substantially reduced compared with the initial ACh response (Fig. 1B), demonstrating that the prior treatment with ACh caused an inhibition of the subsequent Ado response. That the ACh-induced reduction of the Ado response was attributable to the preceding stimulation of M$_2$ mAChR receptors was demonstrated by the observation that in cells lacking the M$_2$ mAChRs, no inhibition of the ACh response by pretreatment with ACh was observed (Fig. 1C). These results showed that prior application of Ado was not responsible for the M$_2$ mAChR-induced desensitization and inhibition of the GIRK currents was demonstrated by results showing similar responses elicited by the M$_2$ mAChRs in cells not previously stimulated with Ado (Fig. 1A and data not shown). The summarized results from a series of similar experiments demonstrated that prior exposure to ACh caused a reduction of the subsequent ACh response to 56 ± 4% (n = 13) of the initial ACh response, whereas prior exposure to Ado only caused a reduction of 5 ± 2.2% (n = 7) (Fig. 1D). The results suggested that activation of the mAChRs caused an initial activation followed by an inhibition of GIRK currents and that the inhibition persisted during a subsequent exposure to Ado and thus diminished the response to Ado.

The Inhibition of Ado-evoked GIRK Currents via Stimulation
Inhibition of GIRK Currents by $M_2$ mAChR

**Fig. 1.** Stimulation of $M_2$ mAChR-induced inhibition of A1 adenosine receptor-mediated activation of GIRK currents. CHO K1 cells transiently transfected with GIRK1, GIRK4, and $M_2$ mAChR and/or A1 adenosine receptors were held in the whole cell-patch clamp configuration, and agonist-activated GIRK currents were measured as inward currents by setting the membrane potential negative (∼−90 mV) to the potassium equilibrium potential of about −50 mV. Illustrated are current recordings that were representative for the indicated conditions. For each panel the horizontal bars at the top of the recordings indicate the duration of application of each agent. The dotted line drawn through the top of the tracings indicates zero current. A, GIRK currents in cells transfected with either the $M_2$ mAChR (left panel) or the A1 adenosine receptors (right panel) were activated in response to laminar superfusion of the cells with either ACh (1 μM, left panel) or Ado (1 μM, right panel). Treatment with ACh caused an initial activation of GIRK currents followed by a pronounced desensitization during a 2-min period of agonist application (left panel). In contrast, a similar treatment with Ado resulted in an activation of the GIRK currents that was followed by a minimal desensitization (right panel). B, in cells expressing both $M_2$ mAChR and A1 Ado receptors, an initial activation by ACh caused a stable activation of GIRK current with minimal desensitization during a 2-min application except for a fast initial, small desensitization. After washout of ACh, currents returned to baseline. Subsequent application of ACh to the same cell caused a transient activation of GIRK currents to a similar magnitude as Ado but was followed by a substantial reduction during the 2-min exposure. After a 1-min washout of ACh, the reappearance of Ado-induced markedly reduced currents compared with the initial Ado response. C, in cells not transfected with $M_2$ mAChR, ACh had no effect alone and no effect on Ado-induced GIRK currents. In addition, prior treatment with Ado did not induce a decrease in the second Ado response. D, summarized data for the reduction of Ado-induced GIRK currents measured 20–60 s after a 2-min application of either ACh or Ado in cells expressing both receptors.

**Fig. 2.** $M_2$ mAChR-mediated inhibition of GIRK currents was slowly reversible. The figure shows a representative experiment in which the experimental protocol was similar to that described in Fig. 1B except that successive pulses of Ado were given following the exposure to and washout of ACh to test the reversibility of the ACh-mediated inhibition of the Ado-evoked currents. The half-time of recovery of the Ado response following the ACh-mediated inhibition was between 2 and 5 min.

A desensitizing treatment with ACh. The cells were co-transfected with the GIRKs and both receptors and initially exposed to a brief pulse of Ado, which activated the currents (Fig. 2). After washout of Ado, the cells were exposed to ACh for 2 min during which there was an initial activation of the GIRK currents to ∼75% of the extent observed with Ado, followed by a substantial inhibition (Fig. 2). Immediately following washout of the ACh the cells were exposed briefly to Ado, and the response was substantially reduced compared with the initial Ado response (Fig. 2). However, the size of the Ado-induced currents increased over time following the washout of ACh, as the subsequent treatments with Ado resulted in larger currents. After 6 min following the washout of ACh, the response to ACh was almost comparable with that of the initial Ado response (Fig. 2, last trace). These results demonstrated that the ACh-induced inhibition of the currents was reversible, albeit with a relatively slow time scale ($t_{1/2} = 2–5$ min).

The $M_2$ mAChR-induced Inhibition of Ado-evoked GIRK Currents Occurs Downstream of Adenosine Receptors—To test whether the ACh-induced inhibition of the currents was due to cross-desensitization of A1 receptors and whether GTP-binding proteins were participating in the inhibitory pathway, we performed experiments in which we activated the currents by preactivating the G proteins with GTPγS added to the pipette solution. This strategy allowed us to eliminate the need to first activate the currents with Ado, thus bypassing the Ado receptor, and allowed us to ask whether ACh could inhibit currents activated directly by G proteins. The amplitude of the GTPγS-activated GIRK currents was determined by a short application of 0.5 mM Ba$^{2+}$, which is sufficient to completely inhibit GIRK currents but does not significantly affect endogenous currents and therefore is used to define the base line (28) (Fig. 3). The difference in currents before and after Ba$^{2+}$ reflected the amplitude of the GTPγS-activated currents, and it can be seen in Fig. 3 that GTPγS caused a substantial activation of the currents. After a complete washout of Ba$^{2+}$ and the return of the currents to the activated state, the cell was superfused with ACh for 1 min. Surprisingly, following an initial small, further activation of the currents, the activation of the $M_2$ mAChRs by ACh caused a marked inhibition of the GTPγS-induced K$^+$ currents (Fig. 3). The extent of inhibition of the GTPγS-activated currents by ACh was 57.5 ± 7.2% ($n = 4$, summarized in Fig. 4C). Moreover, in contrast to experiments performed without GTPγS (for example Fig. 2), no recovery from the ACh-
The Inhibition of GIRK Currents Was Observed in the Presence of Heterologously Expressed Gβγ—To further define where the inhibition of GIRK currents by M2mAChR occurred, we studied the M2mAChR-induced inhibition of GIRK channels that were prestimulated with heterologously expressed Gβγ subunits. CHO cells that were transiently transfected with M2mAChR, GIRK1, GIRK4, and Gβγ subunits exhibited constitutively activated GIRK currents that were blocked by 0.5 mM Ba2+ (Fig. 4A). The currents obtained with Gβγ expression were activated to a much larger degree than with receptor activation or GTPγS (compare Figs. 1, 2, and 3) and presumably reflected full activation of the channels. Consistent with this notion, stimulation of M2mAChR with ACh did not cause any further increase in Ba2+-sensitive GIRK currents (Fig. 4A). Notably, in most cases ACh still caused a reduction of basal currents (Fig. 4A). These ACh-inhibited currents were found to have identical current-voltage relationships as GIRK currents (Fig. 4B), indicating that the Gβγ-induced channel activity was inhibited by stimulation of M2mAChR. However, the ACh-induced inhibition of GIRK currents in the presence of expressed Gβγ was significantly less (20 ± 3.8%, n = 8 after a 2-min application of 1 μM ACh) compared with GIRK currents activated by GTPγS and endogenous G proteins (Fig. 4C). Because these results were obtained under conditions in which full activation of the channels was induced by overexpression of Gβγ, the results suggested that the inhibitory effect of the M2mAChRs on GIRK currents was due to a reduced interaction between the G proteins and the channels. The smaller inhibitory effect observed under these conditions presumably reflected competition between the receptor-induced inhibitory signal and the large pool of active Gβγ subunits.

The Inhibition of GIRK Currents by M2mAChR Was Mediated via Non-PTX-sensitive G Proteins—Because GTP-binding proteins seem to be involved in mediating the inhibitory effect of M2mAChR on GIRK currents (Fig. 3), we tested whether PTX-sensitive G proteins were involved. To do this, transfected CHO cells expressing Gβγ subunits, GIRK1, GIRK4, and M2mAChR were pretreated with 200 ng/ml PTX for 4–6 h and subsequently tested for an ACh-induced inhibition of the Gβγ-activated GIRK currents (Fig. 5). The PTX pretreatment did not prevent or reduce the inhibitory effect of M2mAChR on GIRK currents (inhibition of Ba2+-sensitive GIRK currents after a 2-min incubation with 1 μM ACh was 17.5 ± 6.6%, n = 4). In cells in which Gβγ subunits were not co-expressed, the PTX pretreatment completely blocked activation of GIRK currents by A1 receptors or M2mAChR (n = 4, data not shown),...
Inhibition of GIRK Currents by M<sub>2</sub> mAChR

**Fig. 5.** Pretreatment with PTX did not affect inhibition of G<sub>B</sub>γ-activated GIRK currents. Cells were transfected as described in Fig. 4, and inhibition of G<sub>B</sub>γ-activated GIRK currents by ACh was determined after pretreatment of the cells with PTX (200 ng/ml) for 2–4 h. Note the lack of effect of the PTX treatment on the ACh-mediated inhibition of the G<sub>B</sub>γ-activated currents and the sustained inhibition of currents by ACh even after washout of agonist.

**Fig. 6.** Activation of M<sub>2</sub> mAChRs induced inhibition of A<sub>1</sub> adenosine receptor-activated GIRK currents. Experiments similar to the one described in Fig. 1B were performed using cells that expressed M<sub>4</sub> mAChRs instead of M<sub>2</sub> mAChRs. This representative experiment illustrates the reduction of Ado-activated GIRK currents after superfusion of the cell with 1 μM ACh for 2 min.

indicating that the PTX treatment was 100% effective in blocking receptor-mediated activation of G<sub>i</sub> and G<sub>o</sub> proteins. Thus, the inhibitory effects of the M<sub>2</sub> mAChR on the GIRK channels, in contrast to their stimulatory effects, did not appear to involve a PTX-sensitive G protein. In addition, pretreatment with PTX did not alter the time course of recovery from inhibition after removal of ACh (data not shown).

Can Other GPCRs Inhibit GIRK Currents?—To test whether other GPCRs could also inhibit GIRK currents, M<sub>2</sub> and M<sub>4</sub> mAChRs were tested as well as A<sub>2A</sub> purinergic receptors and α<sub>2A</sub> adrenergic receptors. The M<sub>2</sub> mAChRs, which, like the M<sub>2</sub> mAChRs, are known to couple to PTX-sensitive G proteins, were as effective as M<sub>4</sub> mAChRs in activating GIRK currents and in inducing the inhibition of GIRK currents upon treatment of transfected cells for 2 min with ACh (Fig. 6). Furthermore, activation of the M<sub>4</sub> mAChRs caused an inhibition of a subsequent activation of the A<sub>1</sub> receptors in a manner that was very similar to that caused by the M<sub>2</sub> mAChRs (compare Figs. 1B and 6). In contrast, the other G<sub>i</sub>/G<sub>o</sub>-linked GPCRs tested, namely the α<sub>2A</sub> and α<sub>2C</sub> adrenergic receptors and the A<sub>2A</sub> adenosine receptors, exhibited no obvious inhibitory effect on GIRK currents and basically behaved the same as A<sub>1</sub> adenosine receptors (n = 5, data not shown). On the other hand, activation of M<sub>2</sub> mAChRs, which activate G<sub>i</sub> proteins rather than PTX-sensitive G proteins, caused a strong inhibition of Ado-activated GIRK currents. The inhibition of GIRK currents by the M<sub>2</sub> mAChRs was not blocked by PTX in experiments similar to those described in Fig. 5. To test whether the inhibitory action on GIRK currents was specific for muscarinic receptors, the α<sub>1A</sub> adrenergic receptor (AR), another G<sub>i</sub>-coupled receptor, was tested. Whereas activation of α<sub>1A</sub> ARs with phenylephrine caused a very modest activation of the GIRK currents, the inhibition of the GIRK currents was observed with several subtypes of the mAChRs as well as with the α<sub>1A</sub> adrenergic receptor.

M<sub>2</sub> mAChR-mediated Inhibition of GIRK Currents Was Not Mediated via G<sub>i</sub> Proteins—Because the inhibition of GIRK currents by the G<sub>i</sub>-coupled α<sub>1A</sub> AR or M<sub>2</sub> mAChRs as well as by the normally G<sub>i</sub>/G<sub>o</sub>-coupled M<sub>2</sub> and M<sub>4</sub> mAChRs did not appear to involve PTX-sensitive GTP-binding proteins, we questioned whether the inhibition caused by these receptors utilized a common pathway. Therefore cells transfected with both the M<sub>2</sub>
mACHR and α1A-AR as well as A1 adenosine receptors were pretreated with Pasteurella multocida toxin, a protein toxin that was previously shown to irreversibly uncouple Gs proteins from their receptors, presumably by first activating G proteins and modifying them in a manner that prevented reactivation after deactivation (29, 30).2 After treatment of cells with 1 μg/ml of P. multocida toxin for 20 h, activation of the A1 adenosine receptors was still able to cause activation of the GIRK currents (Fig. 7B). However, co-application of phentolamine did not produce any attenuation of the current, demonstrating that P. multocida toxin treatment resulted in a complete loss of the α1A-AR-induced inhibition of GIRK currents (Fig. 7B). In marked contrast, P. multocida toxin treatment did not affect the inhibition of the GIRK current by the M2 mACHRs, since application of ACh caused a marked inhibition of the Ado-activated GIRK currents (Fig. 7B). These results suggested the existence of two different pathways of GIRK current inhibition, one that is both PTX- and P. multocida toxin-insensitive and induced by the M2 and presumably the M4 mACHRs, and another induced by the Gs-linked α1A-ARs and the M2 mACHRs that can be selectively blocked by P. multocida toxin.

M2 mACHR Inhibited GIRK Currents via a Membrane-delimited Pathway—To determine whether cytosolic soluble messengers were involved in the M2 mACHR-induced inhibition of GIRK currents, we utilized the cell-attached configuration of the patch clamp technique. Channels in the membrane area under the tip of the patch pipette were preactivated with Ado that was included in the patch pipette, and currents were measured in the cell-attached configuration. During the measurement ACh was applied to the bath solution (not to the patch), and effects on the Ado-activated GIRK channel activity were studied. No change in GIRK channel activity was observed in response to the bath application of ACh, suggesting that bath application of ACh was unable to mediate inhibition of the channels being measured under the patch pipette (Fig. 8A). This suggested that ACh was not producing inhibition by generating a soluble second messenger that could theoretically diffuse through the cell to inhibit the channels under the pipette. As a control to demonstrate that the M2 mACHRs were indeed functionally expressed in this cell, 40 min after washout of the bath-applied ACh, the identical cell was measured a second time, now in the whole cell configuration. Under these conditions, a strong inhibition of the Ado-evoked GIRK currents was observed (Fig. 8B), demonstrating that both receptors were functional in this cell and that inhibition by ACh could be observed in the whole cell configuration. These results demonstrated that no cytosolic soluble messenger molecules were needed for the M2 mACHR-mediated inhibition of GIRK currents and that the inhibition occurs via a membrane-delimited pathway.

The M2 mACHR-induced Inhibition of GIRK Currents Was Not Dependent on Internal Calcium Mobilization and Did Not Require Protein Kinases—Recent studies demonstrated that certain Gs-coupled receptors could inhibit GIRK channels expressed in Xenopus oocytes (31, 32). In two studies, contradictory results were reported with regard to the calcium dependence and the role of protein kinases in the Gs-coupled receptor-mediated inhibition of GIRK currents. One study (32) reported that injection of the calcium chelator BAPTA into the oocytes resulted in a decrease of endothelin-1A receptor-induced inhibition of opioid receptor-activated GIRK currents, whereas no effect was found of pretreatment with the non-selective protein kinase inhibitor staurosporine. In contrast, another study (31) reported that there was no calcium dependence but involvement of protein kinase C in the bombesin receptor-mediated inhibition of basal GIRK currents.

We tested whether intracellular Ca2+ or various protein kinases participated in the M2 mACHR-induced inhibition of GIRK currents. Perfusion of cells with 5 mM EGTA and 5 mM BAPTA, which should be sufficient to prevent any substantial rise in the concentration of internal Ca2+, did not prevent the inhibition of Ado-evoked GIRK currents by activation of the M2 mACHR (n = 4, data not shown). This suggested that inositol 1,4,5-trisphosphate-mediated calcium release was not involved in this signaling pathway. Interestingly, the inhibition of GIRK currents by stimulation of α1A adrenergic receptors was not prevented by buffering internal Ca2+ under our conditions. Pretreatment of transfected cells with the non-specific protein kinase inhibitor staurosporine (100 nM for 30–90 min) did change the morphology of the cells but did not have an effect on the M2 mACHR-induced inhibition of Ado-evoked GIRK currents (n = 4, data not shown). Similarly, no effects on the M2 mACHR-mediated inhibition were observed after pretreatments with wortmannin (100 nM, 30–60 min, n = 5) or genistein (0.025 mM, 30–90 min, n = 3). Therefore, we questioned whether the observed inhibition of

2 B. Wilson, personal communication.
Inhibition of GIRK Currents by M2 mACHR

Inhibition of GIRK Currents by M2 mAChRs Was Mediated by PTX-insensitive G Proteins and Occurred Downstream of GPCRs—Under conditions where GIRK channels were constitutively activated either by including GTPγS in the pipette solution or by heterologous expression of Gβγ, stimulation of M2 mAChR resulted in an inhibition of these currents, indicating that this inhibition takes place downstream of GPCRs. Furthermore, the observation that the inhibition of GIRK currents by M2 mAChRs was significantly reduced in the presence of overexpressed Gβγ subunits compared with the inhibition observed following activation of the currents by Ado or GTPγS may point to the G proteins as the target of the M2 mAChR-induced inhibition. Thus, if some factor or event reduces the ability of Gβγ and the GIRKs to interact, the inhibition caused by the hypothetical factor would be predicted to be greater if Gβγ is limiting (which appears to be the case in CHO cells) than under conditions where Gβγ is abundant, as is likely to be the case when Gβγ is overexpressed. However, further studies are required to demonstrate the exact mechanism of the inhibition demonstrated in this study.

The possibility of activating GIRK currents through heterologously expressed Gβγ and of inhibiting the activated currents by stimulation of either M2 or M4 mAChRs allowed us to test whether uncoupling of those receptors from G1 or G2 proteins by PTX had any impact on the inhibition of the GIRK by ACh. Because a PTX pretreatment that resulted in a complete loss of receptor-mediated activation of GIRK currents in cells expressing only endogenous G proteins did not affect the receptor-mediated inhibition of the GIRK currents in cells overexpressing Gβγ, the conclusion can be drawn that the inhibitory effect of the M2 mAChR was not mediated through PTX-sensitive G proteins. However, other yet to be identified GTP-binding proteins may be involved in the pathway that links M2 mAChR to the inhibition of GIRK currents. The M2 mAChR-induced inhibition of Ado-evoked GIRK currents was at least partially reversible (Fig. 2); however, the inhibition of GTPγS-preactivated currents was not reversible (Fig. 3). Because PTX-sensitive G proteins do not mediate the inhibitory effect of M2 mAChR on GIRK currents, the conversion of the inhibitory receptors as well as via α2A and α2C adrenergic receptors, which, like the M2 mAChR, activate GIRK channels via PTX-sensitive G proteins, did not cause an inhibition of GIRK currents. However, the M4 mAChRs, which also couple to PTX-sensitive G proteins, caused effects similar to those of the M2 mAChR. These results indicated that the subtypes of the mAChR that couple to G/Gbg proteins induced an additional inhibitory signaling pathway, which was not activated by the M2 mAChR and A1 adenosine receptors.

The Inhibition of GIRK Currents by M2 mAChRs Was Independent of Receptor Phosphorylation and Internalization—The M2 mAChRs are known to internalize in a phosphorylation-dependent manner within a few minutes of agonist treatment (21, 37). In contrast, A1 adenosine receptors require much longer agonist treatment to internalize into intracellular compartments (34, 35). Whereas phosphorylation of the M2 mAChR is required for homologous desensitization (21, 36) and mutants of the M2 mAChR that are impaired in agonist-induced phosphorylation have been demonstrated to be severely impaired in their ability to internalize in an agonist-dependent manner (21, 37), the results obtained in this study demonstrate that a phosphorylation-deficient mutant of the M2 mAChR is fully able to cause inhibition of the GIRK currents. Therefore, the inhibitory action of the M2 mAChRs on Ado-evoked GIRK currents was independent of internalization and phosphorylation of the receptors.

Inhibition of GIRK Currents by Muscarinic Receptors Was Mediated by PTX-insensitive G Proteins and Occurred Downstream of GPCRs—Under conditions where GIRK channels were constitutively activated either by including GTPγS in the pipette solution or by heterologous expression of Gβγ, stimulation of M2 mAChR resulted in an inhibition of these currents, indicating that this inhibition takes place downstream of GPCRs. Furthermore, the observation that the inhibition of GIRK currents by M2 mAChRs was significantly reduced in the presence of overexpressed Gβγ subunits compared with the inhibition observed following activation of the currents by Ado or GTPγS may point to the G proteins as the target of the M2 mAChR-induced inhibition. Thus, if some factor or event reduces the ability of Gβγ and the GIRKs to interact, the inhibition caused by the hypothetical factor would be predicted to be greater if Gβγ is limiting (which appears to be the case in CHO cells) than under conditions where Gβγ is abundant, as is likely to be the case when Gβγ is overexpressed. However, further studies are required to demonstrate the exact mechanism of the inhibition demonstrated in this study.

The possibility of activating GIRK currents through heterologously expressed Gβγ and of inhibiting the activated currents by stimulation of either M2 or M4 mAChRs allowed us to test whether uncoupling of those receptors from G1 or G2 proteins by PTX had any impact on the inhibition of the GIRK by ACh. Because a PTX pretreatment that resulted in a complete loss of receptor-mediated activation of GIRK currents in cells expressing only endogenous G proteins did not affect the receptor-mediated inhibition of the GIRK currents in cells overexpressing Gβγ, the conclusion can be drawn that the inhibitory effect of the M2 mAChR was not mediated through PTX-sensitive G proteins. However, other yet to be identified GTP-binding proteins may be involved in the pathway that links M2 mAChR to the inhibition of GIRK currents. The M2 mAChR-induced inhibition of Ado-evoked GIRK currents was at least partially reversible (Fig. 2); however, the inhibition of GTPγS-preactivated currents was not reversible (Fig. 3). Because PTX-sensitive G proteins do not mediate the inhibitory effect of M2 mAChR on GIRK currents, the conversion of the inhibitory

GIRK currents, which is induced by M2 mAChR but not by A1 and A2 adenosine receptors, is mechanistically connected to the phosphorylation and internalization of muscarinic receptors. Mutants of the M2 mAChR that either lack the middle part of the third intracellular loop (M2Δ1 mAChR) (21) or in which two clusters of serine or threonine residues in that loop are mutated to alanines (36) are unable to undergo agonist-induced phosphorylation and are severely impaired in their ability to internalize. We utilized these mutants to test whether or not the inhibition of GIRK currents by the M2 mAChR depends on the agonist-induced phosphorylation and internalization of these receptors. When cells were transiently transfected with M2Δ1 mAChR, A1 adenosine receptors, and GIRK1 and GIRK4, ACh rapidly induced inwardly rectifying K+ currents and caused inhibition of subsequent adenosine-evoked GIRK currents (Fig. 9). No difference was found between the ability of the M2Δ1 and wild-type M2 mAChRs to mediate the inhibition of the GIRK currents. Therefore, the inhibitory effects on the GIRK currents do not depend on either agonist-mediated phosphorylation of the receptors or on the internalization of M2 mAChRs.

DISCUSSION

Inhibition of GIRK Currents Was Mediated through Muscarinic but Not A1 or A2 Purinergic Receptors—The M2 mAChRs are known to activate several signaling systems, including GIRK channels, via PTX-sensitive G proteins. In the present study demonstrated that M2 mAChR and M4 mAChR not only activated GIRK currents but also inhibited GIRK currents activated via themselves, A1 adenosine receptors, or directly by G proteins. The inhibitory effect of ACh on GIRK currents followed a much slower time course (t1/2 = 20-50 s) than the activation of GIRK currents, which peaks after a few seconds (t1/2 = 0.2-1 s) (19), and therefore might represent a mechanism of heterologous desensitization. ACh neither elicited GIRK currents nor inhibited Ado-activated GIRK currents when no muscarinic receptor was co-expressed, indicating that both the activation as well as the subsequent inhibition of GIRK currents specifically required activation of the heterologously expressed mAChRs. Prolonged activation of GIRK currents via A1 adenosine receptors or A2 adenosine

FIG. 9. The inhibition of GIRK currents did not require agonist-induced phosphorylation or internalization of muscarinic receptors. Cells were transfected with cDNAs for GIRK1/4, A1 Ado receptor, and a mutant M2 mAChR (M2Δ1 mAChR, lacking a significant portion of its third intracellular loop) that has been shown to be deficient in agonist-induced phosphorylation and internalization (21). GIRK currents were measured as described in Fig. 1B. ACh caused activation and then inhibition of the currents during the 2-min exposure as well as the subsequent inhibition of Ado-induced currents.
effect of the M₂ mAChR from a reversible to an irreversible effect in the presence of the slowly hydrolyzable GTP analog suggests that other GTP-binding proteins must be involved. A potential candidate might be Gₛ, which is a member of the Gₛ/G₁₁ family but PTX-insensitive.

Gₛ Protein-coupled Receptors Inhibited GIRK Currents through a Different Pathway—ACh-induced muscarinic K⁺ currents can be inhibited via α₁ adrenergic receptors in atrial myocytes (38), most likely via activation of Gₛ proteins and production of soluble second messengers. We present in this study two major pieces of evidence that M₂ mAChRs do not cause production of soluble second messengers. We present in this study. Furthermore, we found that the M₂ mAChR-inhibited GIRK currents can be blocked by α₁-adrenergic receptors, bombesin, or endothelin 1A receptors (31, 32, 39). In Xenopus oocytes the endothelin-induced inhibition of μ-opioid receptor-activated GIRK currents through endothelin 1A receptors was partially sensitive to Ca²⁺-chelating agents (BAPTA) but not affected by protein kinase inhibitors such as staurosporine (32). Inhibitors of calcium-dependent phospholipase A₂ caused a 40% reduction in the inhibitory effect of endothelin 1A receptors at the highest phospholipase A₂ inhibitor concentration used. Because arachidonic acid can be generated through phospholipase A₂ activity and because extracellularly applied arachidonic acid inhibited GIRK currents, Rogalski et al. (32) postulated that endothelin 1A receptors inhibited GIRK currents through mobilization of intracellular Ca²⁺ and subsequent activation of phospholipase A₂ and generation of arachidonic acid. In contrast to this finding, in another study it was concluded that metabotropic glutamate receptors inhibited basal and ACh-activated GIRK currents through activation of a staurosporine- and bisindolylmaleimide-sensitive kinase that may have been protein kinase C because phospholipase C was able to mimic this effect (39). Similar results were reported for the inhibition of basal GIRK currents through activation of phosphoinositide metabolism (31). Whereas each of these studies found that Gₛ-coupled receptors can cause inhibition of GIRK currents in Xenopus oocytes, the contradictory results concerning the inhibitory mechanism(s) will require future studies to determine the exact pathway(s) that mediates inhibition of GIRK currents through Gₛ-coupled receptors. The M₂ mAChRs did not mediate their inhibitory effect via Gₛ since the effect was not blocked by P. multocida toxin, whereas this toxin did block the action of the Gₛ-coupled receptors tested in this study. Furthermore, we found that the M₂ mAChR-induced inhibition of GIRK currents was insensitive to staurosporine, H-7, wortmannin, genistein, and chelation of intracellular Ca²⁺ with BAPTA and EGTA, indicating that the pathway utilized by the M₂ mAChR receptor is different from the pathway that mediates the inhibition of GIRK currents by Gₛ-coupled receptors. Recently, another study described that dopamine D₂ dopaminergic receptors and somatostatin SST1 receptors can activate homomeric GIRK2 channels via PTX-sensitive G proteins in CHO cells. Interestingly, activation of the D₂ dopamine receptors induced an attenuation of the somatostatin response but not vice versa (40), suggesting that the D₂ dopamine receptors may act by a pathway that is similar to that used by the M₂ and M₄ mAChRs to cause inhibition of GIRK currents.

Further evidence that some receptors that couple to PTX-sensitive G proteins can induce inhibition of other Gₛ/G₁₁-coupled pathway proteins came from a study that compared the inhibition of N-type Ca²⁺ channels by k- and μ-opioid receptors and GABA B receptors. Results from this study indicated that agonists of k- and μ-opioid receptors inhibited N-type Ca²⁺ currents to a lesser extent than did GABA B receptors, but if opioid agonists were added together with GABA B receptors, the opiate agonists attenuated the GABA B response (2). It is unclear if this cross-talk is related to the inhibition of Ado-evoked GIRK currents by muscarinic receptors, but these studies and our results presented here clearly demonstrate that signaling of G protein-coupled receptors that are known to couple to the same family of G protein-coupled receptors is more divergent and complex than previously anticipated. Further studies will be required to identify the components that mediate the inhibition of the GIRK channels by the receptors described here and to ascertain whether the inhibitory signals are specific for the GIRK channels or whether they participate in regulating other downstream effectors of these GPCRs.

REFERENCES

1. Strader, C. D., Fouq, T. M., Graziano, M. P., and Tota, M. R. (1995) FASEB J. 9, 745–754
2. Polo-Parada, L., and Pilar, G. (1999) J. Neurosci. 19, 5213–5227
3. Sakmann, B., Noma, A., and Trautwein, W. (1983) Nature 304, 250–253
4. Belardinelli, L., and Isenberg, G. (1988) Am. J. Physiol. 255, H734–H737
5. Búnnemann, M., Liilim, K., Brands, B. K., Pott, L., Tseng, J. L., Desiderio, D. M., Sun, G., Miller, D., and Tucy, G. (1996) EMBO J. 15, 5527–5534
6. Soejima, M., and Noma, A. (1984) Pfluegers Arch. Eur. J. Physiol. 400, 424–431
7. Breitwieser, G. E., and Szabo, G. (1985) Nature 317, 538–540
8. Dascal, N. (1997) Cell. Signal. 9, 551–573
9. Wickman, K., and Clapham, D. E. (1995) BioTechniques 23, 865–885
10. Logothetis, D. E., Kurachi, Y., Galper, E. J., and Clapham, D. E. (1987) Nature 325, 321–324
11. Krapivinsky, G., Krapivinsky, L., Wickman, K., and Clapham, D. E. (1995) J. Biol. Chem. 270, 29059–29062
12. Krapivinsky, G., Kennedy, M. E., Neneec, J., Medina, I., Krapivinsky, L., and Clapham, D. E. (1998) J. Biol. Chem. 273, 16946–16952
13. Krapivinsky, G., Gordon, A. E., Wickman, K., Velimirovic, B., Krapivinsky, L., and Clapham, D. E. (1995) Nature 374, 135–141
14. Corey, S., Krapivinsky, G., Krapivinsky, L., and Clapham, D. E. (1998) J. Biol. Chem. 273, 5271–5278
15. Lesage, F., Duprat, F., Fink, M., Guillemare, E., Coppola, T., Lazdunski, M., and Hugnot, J. P. (1994) FEBS Lett. 353, 37–42
16. Kofuj, I., Davidson, N., and Lester, H. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6542–6546
17. Kubo, Y., Reuvens, E., Sleisinger, P. A., Jan, Y. N., and Jan, L. Y. (1993) Nature 364, 802–806
18. Dascal, N., Schreibmayer, W., Lim, N. F., Wang, W., Chavkin, C., DiMagno, L., Labarca, C., Kieffer, B. L., Gaveriaux-Ruff, C., Trollinger, D., Lester, H. A., and Davidson, N. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10235–10239
19. Bünemann, M., and Hoey, M. S. (1998) J. Biol. Chem. 273, 31186–31190
20. Jurman, M. E., Boland, L. M., Liu, Y., and Yellen, G. (1994) BioTechniques 17, 876–881
21. Paal-Rylaarsdam, R., Xu, Y., Witt-Endeber, P., Benovic, J. L., and Hoezy, M. M. (1995) J. Biol. Chem. 270, 29004–29011
22. Forsayth, J. R., and Garcia, P. D. (1994) BioTechniques 17, 354–358
23. Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981) J. Physiol. 34, 43–50
24. Bünemann, M., Brands, B., zu Heringdorf, D. M., van Koppen, C. J., Jakobs, K. H., and Pott, L. (1995) J. Physiol. (Lond.) 489, 711–777
25. Chuang, H. H., Yu, M., Jan, Y. N., and Jan, L. Y. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11727–11732
26. Vorobiev, D., Levin, G., Lotan, I., and Dascal, N. (1998) Pfluegers Arch. Eur. J. Physiol. 436, 56–68

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27. Shui, Z., Boyett, M. R., and Zang, W. J. (1997). *J. Physiol. (Lond.)* **505**, 77–93.
28. Nichols, C. G., and Lopatin, A. N. (1997). *Annu. Rev. Physiol.* **59**, 171–191.
29. Wilson, B. A., Zhu, X., Hu, M., and Lu, L. (1997). *J. Biol. Chem.* **272**, 1268–1275.
30. Wilson, B. A., Puñerrada, V. G., Vallance, J. E., and Ho, M. (1999). *Infect. Immun.* **67**, 80–87.
31. Stevens, E. B., Shah, B. S., Pinnock, R. D., and Lee, K. (1999). *Mol. Pharmacol.* **55**, 1020–1027.
32. Rogalski, S. L., Cyr, C., and Chavkin, C. (1999). *J. Neurochem.* **72**, 1409–1416.
33. Pals-Rylaarsdam, R., Gurevich, V. V., Lee, K. B., Ptasienski, J. A., Benovic, J. L., and Hosey, M. M. (1997). *J. Biol. Chem.* **272**, 23682–23689.
34. Nie, Z., Mei, Y., and Ramkumar, V. (1997). *Mol. Pharmacol.* **52**, 456–464.
35. Ciruela, F., Saura, C., Canela, E. I., Mallol, J., Lluis, C., and Franco, R. (1997). *Mol. Pharmacol.* **52**, 788–797.
36. Pals-Rylaarsdam, R., and Hosey, M. M. (1997). *J. Biol. Chem.* **272**, 14152–14158.
37. Bunemann, M., Lee, K. B., Pals-Rylaarsdam, R., Roseberry, A. G., and Hosey, M. M. (1999). *Annu. Rev. Physiol.* **61**, 169–192.
38. Braun, A. P., Fedida, D., and Giles, W. R. (1992). *Pfluegers Arch. Eur. J. Physiol.* **421**, 431–439.
39. Sharon, D., Vorobiev, D., and Dascal, N. (1997). *J. Gen. Physiol.* **109**, 477–496.
40. Kuzhikandathil, E. V., Yu, W., and Oxford, G. S. (1998). *Mol. Cell. Neurosci.* **12**, 390–402.
Novel Inhibition of Gβγ-activated Potassium Currents Induced by M2 Muscarinic Receptors via a Pertussis Toxin-insensitive Pathway
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