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Role of the Small GTPase Rho in Modulation of the Inwardly Rectifying Potassium Channel Kir2.1.

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ABSTRACT

The inwardly rectifying potassium channel Kir2.1 is inhibited by a variety of G-protein-coupled receptors (GPCRs). However, the mechanisms underlying the inhibition have not been fully elucidated. In this study the role of the small GTPase, Rho, in mediating this inhibition was determined. Stimulation of the m1 muscarinic receptor inhibited Kir2.1, when both receptor and channel were coexpressed in tsA201 cells. The inhibition of Kir2.1 by carbachol was reversible and atropine-sensitive. Cotransfection with a dominant-negative mutant of the small GTPase Rho abolished the inhibition of Kir2.1 with current amplitudes remaining at control levels in the presence of carbachol. Conversely, cotransfection with the constitutively activated mutant of Rho resulted in a reduction in basal Kir2.1 current amplitudes, suggesting that Rho inhibits Kir2.1. To further confirm the involvement of Rho in the signal transduction pathway, cotransfection with C3 transferase (EFC3), a selective inhibitor of Rho, abolished the reduction in Kir2.1 currents noted upon application of carbachol under control conditions. Preincubation with the phosphatidylinositol 3-kinase inhibitor wortmannin or the Rho kinase inhibitor (*R*)−(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide, 2 HCl (Y-27632) had no effect on agonist-induced inhibition of Kir2.1, precluding these kinases as downstream effectors of Rho in mediation of the signal. In addition, 2′-amino-3′-methoxyflavone (PD98059), an inhibitor of mitogen-activated protein (MAP) kinase kinase (MEK), had no effect on the m1 receptor-induced inhibition of Kir2.1, suggesting that MAP kinases are not involved in the signaling pathway. In conclusion, these data indicate that the small GTPase, Rho, transduces the m1 muscarinic receptor-induced inhibition of Kir2.1 via an unidentified mechanism.

Inwardly rectifying potassium channels are involved in a multitude of responses, including control of heart rate, neuronal firing patterns, vascular smooth muscle tone, secretion of hormones, and activation of immune response cells. They are important for maintenance of the resting membrane potential of a cell near the potassium equilibrium potential. Modulation of this type of channel can result in profound changes in the electrical excitability of the cell. Activation of these channels will hyperpolarize the membrane potential of the cell, whereas inhibition of their activity can result in a robust depolarization of the membrane. Inwardly rectifying potassium channels comprise a large group of potassium channels that have been cloned, revealing a far greater heterogeneity of channel subtype than expected. The channels can be divided into seven classes designated as Kir 1 to 7 (reviewed by Stanfield et al., 2002). The Kir2.x or IRK type of inwardly rectifying potassium channels Kir2.1 to 2.4 are strong rectifiers, are constitutively active, and are expressed throughout the body (Stanfield et al., 2002).

Several studies have demonstrated that strongly rectifying constitutively active inward rectifiers similar to Kir2.x family members are inhibited by activation of GPCRs in native tissue. This inhibition has been noted in central nervous system neurons by stimulation of muscarinic (Uchimura and North, 1990) and substance P receptors (Takano et al., 1995), in ventricular myocytes (Fedida et al., 1991; Wang et al., 2001) and glial cells (Roy and Sontheimer, 1995) by α-adrenergic receptor stimulation, in adrenal glomerulosa cells by angiotensin II (Kanazirska et al., 1992), in macrophages by acetylcholine (Moody-Corbett and Brehm, 1987), and in endothelial cells by endothelin-1 (Zhang et al., 1994). Because this activity seems to be quite widespread, determining the mechanisms by which these channels are modulated will further our understanding of control of a variety of cellular functions.

We have shown previously that members of the Kir2.x family are inhibited by muscarinic receptor stimulation (Jones, 1996, 1997; Firth and Jones, 2001; T. M. Rossignol, T. A. Firth, and S. V. P. Jones, submitted for publication), when expressed in mammalian cells cotransfected with the

ABBREVIATIONS: GPCR, G-protein-coupled receptor; PKC, protein kinase C; PKA, protein kinase A; MAPK, mitogen-activated protein kinase; GFP, green fluorescence protein; GEF, guanine nucleotide exchange factor; PD98059, 2′-amino-3′-methoxyflavone; NMS, *N*-methylscopolamine; EFC3, exchange factor C3; PMA, phorbol 12-myristate-13-acetate; ROK, Rho kinase; PI(3)K, phosphatidylinositol 3-kinase; PI(4,5)P_2, phopshatidylinositol-4,5-bisphosphate; MEK, mitogen-activated protein kinase kinase; IGF, insulin-like growth factor; PDZ, postsynaptic density 95/disc-large/ZO-1 domain Y-27632, (R)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide, 2 HCl.

m1 receptor and Kir2.x genes. The mechanism by which the m1 muscarinic receptor inhibited Kir2.1 has not been fully elucidated. However, Kir2.1 currents are inhibited by activation of protein kinase C (PKC) (Fakler et al., 1994; Jones, 1996, 1997; T. M. Rossignol, T. A. Firth, and S. V. P. Jones, submitted for publication). In some studies Kir2.1 was also inhibited by cAMP-dependent protein kinase (PKA) (Wischmeyer and Karschin, 1996) and by cAMP (Jones, 1997), whereas other studies noted either no effect (Jones, 1996) or an increase in Kir2.1 current amplitude (Fakler et al., 1994). The inhibition of Kir2.1 by PKA may be caused by the dissociation of Kir2.1 from a cytoskeletal protein, postsynaptic density protein-95, which has been shown to combine with Kir2.x channels and is dissociated from them by phosphorylation of the PKA phosphorylation site (Cohen et al., 1996). More recently, phosphatidylinositol-4,5-bisphosphate has been shown to interact with members of the Kir2.x family (Huang et al., 1998; Rohacs et al., 1999; Zhang et al., 1999). Also, some members of the Kir2.x family have been shown to be modulated by pH (reviewed by Lopatin and Nichols, 2001), magnesium (Chuang et al., 1997) and tyrosine (Wischmeyer et al., 1998; Tong et al., 2001), and mitogen-activated protein kinases (MAPKs) (Giovannardi et al., 2002).

Recently several G-protein-coupled receptors, including the m1 muscarinic receptor, have been shown to activate pathways involving the small GTPases, such as the Rho-like family of small G-proteins, which include Rho, Rac, and Cdc42 (Sah et al., 2000; Chikumi et al., 2002). The effects of the small GTPases on ion channels are largely unknown. However, Kv1.2, a delayed rectifier potassium current that is inhibited by muscarinic receptor stimulation, was inhibited by tyrosine kinase phosphorylation via a mechanism that also involved Rho A (Cachero et al., 1998). Wilk-Blaszczak et al. (1997) showed that Rac 1 and Cdc42 are required for inhibition of a voltage-dependent calcium channel by bradykinin. More recently, a volume-activated anion channel (Nilius et al., 1999) and an ether-a-go-go channel (Storey et al., 2002) have been shown to be regulated by Rho. Therefore, this study was performed to determine the role of the small GTPases in mediating inhibition of Kir2.1 by the m1 muscarinic G-protein-coupled receptor.

Materials and Methods

Cell Culture and Transfection Procedures. The tsA201 cells (also known as human embryonic kidney 293T cells) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C and in 5% CO_2.

The cells were transiently transfected with 5 μg of each of four cDNAs by the calcium phosphate precipitation method. The control cells were transiently cotransfected with 5 μg of Kir2.1 cDNA, 5 μg of m1 cDNA, 5 μg of GFP, and 5 μg of the neomycin resistance gene. The use of empty vector as a control is valid in maintaining consistent cDNA concentrations. However, the expression levels of Kir2.1 and receptor may be affected by expression of a fourth protein; thus, it is essential to include a fourth, but unrelated protein for expression under control conditions. The test cells were cotransfected with 5 μg each of the Kir2.1, m1, GFP, and small G-protein cDNAs. The cells were recorded from for 2 to 3 days after transfection. To reduce bias in the data caused by variability in the transfections, several separate transfections were performed (four to five) for each set of conditions, and several cells were tested from each transfection. Control and test transfections were performed in pairs and the experiments were conducted simultaneously. For example, control cells and cells transfected with the dominant-negative mutant of Rho and the dominant-negative mutant of Rac were recorded from in random sequence on the same day. This reduced any bias caused by differences in recording conditions, such as the temperature or pipette conformation.

DNA Constructs. The m1 muscarinic receptor was inserted into the vector pcD as reported previously (Jones, 1996). Kir2.1 was inserted into the vector pcDNA1 (Invitrogen, Carlsbad, CA) as reported previously (Kubo et al., 1993). Dr. Lily Y. Jan (University of California, San Francisco, CA) kindly provided Kir2.1 (Kubo et al., 1993). The activated mutant forms of Rac and Rho were kindly provided by Dr. Silvio Gutkind (National Institute of Dental & Craniofacial Research, National Institutes of Health, Bethesda, MD) and were generated by replacing glutamine for leucine by polymerase chain reaction-directed mutagenesis in a position analogous to that of codon 61 of Ras, generating Rac1-QL at codon 61 and RhoA-QL at codon 63 as described by Coso et al. (1995). Such a mutation has been shown to inhibit the GTPase activity of these proteins, resulting in a constitutively active protein. The dominant-negative forms of the small G-proteins Rac and Rho, also kind gifts from Dr. Silvio Gutkind, were synthesized by replacement of the amino acid threonine for asparagine in a position analogous to codon 17 of Ras, generating N17-Rac1 and N19-RhoA. These inhibitory mutants can act as antagonists by competitively inhibiting the interaction of endogenous G-proteins with their respective guanine nucleotide exchange factors (GEFs) and blocking transduction of the signal (Coso et al., 1995; Hooley et al., 1996; Lamaze et al., 1996). Transfections with N17-Ras and N17-Rac1 or N17-Cdc42 have been shown to inhibit downstream activation of MAPK and c-Jun NH_2-terminal kinase, respectively by epidermal growth factor (Coso et al., 1995), indicating their effectiveness as inhibitors. N19-RhoA did not inhibit this activity, indicating the specificity of these mutants as reagents. N19-RhoA has been shown to effectively inhibit endocytosis (Lamaze et al., 1996) and Na-H exchange (Hooley et al., 1996).

Dr. Richard Treisman (London Research Institute, Lincoln's Inn Fields, London, UK) kindly provided the C3 exoenzyme plasmid. This plasmid encodes the C3 toxin that ADP-ribosylates Rho in a manner analogous to that of pertussis toxin (Hill et al., 1995).

Electrophysiological Recordings. Kir2.1 whole cell currents were recorded using a List EPC-7 patch-clamp amplifier (List Electronics, Darmstadt, Germany), as described previously (Jones, 1996, 1997; Firth and Jones, 2001). All experiments were carried out at room temperature. Electrodes were pulled on a three-stage horizontal puller (Mecanex, Nyon, Switzerland) and fire polished. Electrodes had resistances of 4 to 7 MΩ. Currents were low pass-filtered at 1 kHz with an eight-pole Bessel filter, digitized through an ITC-16 analog-digital converter (InstruTECH Corporation, Port Washington, NY), and sampled at 2 kHz with a Macintosh Quadra 800 computer (Apple Computer, Cupertino, CA). Currents were acquired with Axodata (Axon Instruments, Inc., Union City, CA). Series resistance and capacitance compensation were applied to the current responses.

Kir2.1 currents were elicited by holding the cells in whole-cell voltage clamp at a potential of −60 mV and stepping the membrane to potentials ranging from −160 to 40 mV for 200 ms in 10-mV increments as described previously (Jones, 1996, 1997). This protocol was repeated every 2 min for the duration of the experiment. After initial breakthrough into the whole-cell mode, a 10-min period was allowed for stabilization of the Kir2.1 current amplitude before application of agonist. Carbachol or acetylcholine was applied at a concentration of 50 μM from a flow pipette. The responses to agonist were followed for 10 to 20 min to ensure the full effect, and then the drug was washed out with fresh extracellular solution. Recovery from the actions of agonist was followed over the next 10 min.

Data Analysis. Currents were analyzed off-line, using Axograph software (Axon Instruments, Inc.). For the inwardly rectifying currents, measurements were determined by averaging the last 10 data

points of the 200-ms step. For current-voltage curves, the current amplitudes were plotted against the voltage that the cell membrane potential was stepped to. Statistical significance was determined using nonparametric statistical methods. Data are presented as mean ± S.E.

Recording Solutions and Drugs. The extracellular recording solution was composed of 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, and 20 mM glucose, pH corrected to 7.4 with 1 M NaOH. The osmolarity of the solution was adjusted to 325 to 330 mmol/kg. The intracellular patch pipette solution contained 150 mM potassium-gluconate, 2 mM $MgCl_2$, 1.1 mM EGTA, 0.1 mM $CaCl_2$, 5 mM HEPES, 5 mM Mg-ATP, and 0.1 mM Li-GTP. The pH was adjusted to 7.2 and the osmolarity to 315 to 320 mmol/kg.

Y-27632, wortmannin, and PD98059 were obtained from Calbiochem (San Diego, CA). PD98059 and wortmannin were dissolved in dimethyl sulfoxide. Final concentrations of dimethyl sulfoxide were 0.4% and 1% for PD98059 and wortmannin, respectively.

Ligand Binding Assay. Muscarinic receptor binding sites were determined as described previously (Jones, 1996). Briefly, cell membranes were collected and homogenized in sodium phosphate buffer containing 25 mM HEPES and 5 mM $MgCl_2$, pH 7.4. Binding assays were initiated by addition of 900 μl of membranes to 100 μl of ligand. Membranes were incubated for 2 h at room temperature, and specific *N*-[^{3}H]methylscopolamine binding was measured. Nonspecific binding was determined in the presence of 1 μM atropine.

Results

Muscarinic Receptor-Induced Inhibition of Kir2.1. tsA201 cells were transfected with Kir2.1, m1, GFP, and neomycin resistance gene. The resultant currents were typical of Kir2.1 with fast activation and deactivation kinetics (Fig. 1A). Stimulation of the m1 muscarinic receptor with the agonist carbachol resulted in a 54 ± 6% (n = 9) inhibition of the current amplitude (Fig. 1A), as we have noted previously (Jones, 1996; Firth and Jones, 2001). On washout of the carbachol, inwardly rectifying currents returned to near control values. The response to stimulation of the m1 muscarinic receptor was atropine-sensitive, confirming the muscarinic nature of the response (Figs. 1B and 2).

Involvement of Small G-Proteins in Modulation of Kir2.1. To identify the roles of the various small G-proteins, mutants of many of the proteins have been expressed in cell lines (Coso et al., 1995). These include the constitutively activated and dominant-negative mutants of the small G-proteins. The activated forms of Ras, Rho, Rac, and Cdc42 have been shown to transduce their effects in the absence of activators, such as GPCRs or tyrosine kinase receptors. Likewise, the dominant-negative mutants of Ras, Rho, Rac, and Cdc42 have been used as antagonists, selectively inhibiting the activity of their endogenous counterparts.

To investigate the possible role of the small G-proteins in inhibition of Kir2.1, we initially tested the dominant-negative mutants of Rac and Rho. Cotransfection of the tsA cells with the dominant-negative mutant of RhoA (N19-RhoA), Kir2.1, GFP, and m1 abolished the inhibition of Kir2.1 by the m1 muscarinic receptor (Fig. 2). The ~50% inhibition induced by carbachol under control conditions was reduced to 1% in cells cotransfected with N19-RhoA. In contrast, cotransfection of the cells with the dominant-negative mutant of Rac1 (N17-Rac1) had no effect on the m1 muscarinic receptor-induced inhibition of Kir2.1.

Because Rho is known to have multiple effects on cell function, it is possible that the inhibition of the muscarinic response is caused by the down-regulation of muscarinic receptors by the dominant-negative mutant of RhoA. To test this, we measured the m1 muscarinic receptor expression levels, using *N*-[^{3}H]methylscopolamine (NMS) binding. Comparisons were made between cells transfected under control conditions (Kir 2.1, m1, GFP, and neomycin resistance gene) and cells coexpressing N19-RhoA (Kir 2.1, m1, GFP, and N19-RhoA).

The results indicated that the levels of m1 muscarinic receptor were not significantly altered by cotransfection with N19-RhoA. Control [^{3}H]NMS binding sites were 598 ± 137 fmol/mg protein (n = 3). [^{3}H]NMS binding in cells cotransfected with N19-RhoA was 652 ± 94 fmol/mg protein (n = 3).

Further confirmation of the effects of the dominant-negative mutant of Rho was obtained by using the C3 exoenzyme, an inhibitor of Rho, using the C3 plasmid (EFC3) (Hill et al., 1995). Cotransfection with EFC3 reduced the carbachol-induced inhibition from ~50% in control cells to 3% inhibition in cells that were transfected with EFC3 (Fig. 2). Thus, the effects of cotransfection with EFC3 in tsA cells expressing

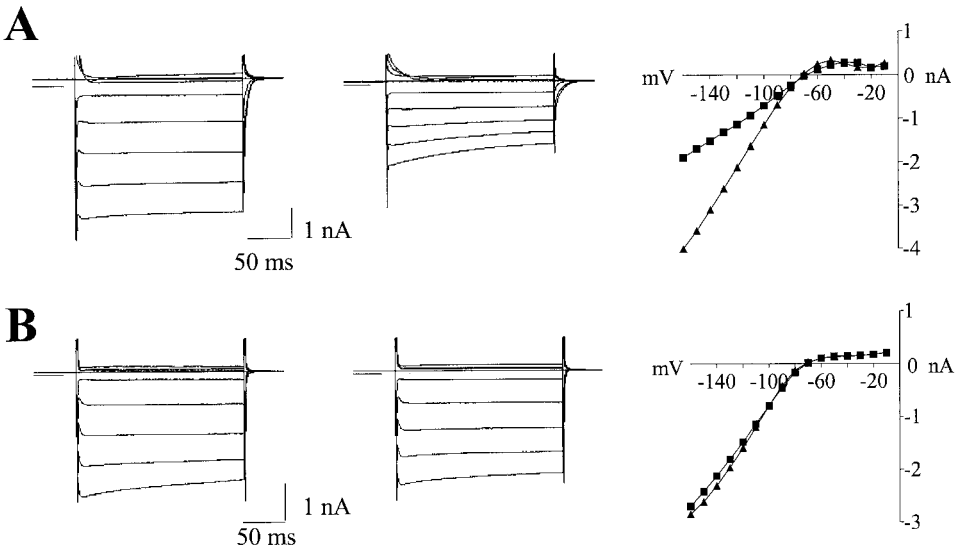

Fig. 1. Carbachol induced an inhibition of the inwardly rectifying currents in tsA cells transfected with Kir 2.1 and the m1 muscarinic receptor. The superimposed currents were elicited by 200-ms steps from −60 mV to potentials ranging from −160 to −20 mV in 20-mV increments. Left, representative currents under control conditions; middle, currents recorded from the same cell during application of 50 μM carbachol from a flow pipette. The bars on the left side of the traces indicate zero current levels. Right, current-voltage relationships from the same cell. A, control conditions; B, recordings from a different cell incubated with 1 μM atropine.

Kir2.1, m1, and GFP further confirmed the role of Rho in the muscarinic receptor-induced inhibition of Kir2.1.

The effects of the activated mutant of Rho (RhoA-QL) were also examined. Comparisons were made with control tsA cells cotransfected with Kir2.1, m1, GFP, and neomycin resistance and cells cotransfected with Kir2.1, m1, GFP, and RhoA-QL. The Kir2.1 current amplitudes in cells that were transfected with RhoA-QL were significantly smaller than those recorded in control cells (Fig. 3). However, because transfection with RhoA-QL resulted in small spherical cells, the data were converted to current density (pA/pF) to take differences in cell size into account, using cellular capacitance. In cells cotransfected with N19-RhoA, the Kir2.1 current density was not significantly different from the current density observed in control cells. However, in cells that had been cotransfected with RhoA-QL, the current density was significantly reduced, indicating that the constitutively active Rho inhibited Kir2.1 (Fig. 3).

Previously, we have shown that activation of PKC by phorbol esters inhibited Kir2.1 (Jones, 1996). Therefore, the role of PKC in the signal transduction pathway mediated via Rho was studied. First, the effects of phorbol 12-myristate-13-acetate (PMA) were compared in cells that had been cotransfected with EFC3 with control cells. Under control conditions, 1 μM PMA induced a 29 $\pm$ 3% (n = 7) inhibition of Kir2.1 similarly to that observed previously (Jones, 1996). However, in cells that were transfected with EFC3, the response to PMA was abolished. Kir2.1 current amplitudes remained at control levels of 106 $\pm$ 4% (n = 6) in the presence of 1 μM PMA. In addition, the effects of inhibiting PKC by calphostin C were determined in cells that were transfected with activated Rho, RhoA-QL. Calphostin C had been shown previously to inhibit the m1 muscarinic receptor-induced inhibition of Kir2.1 (Jones, 1996). Preincubation of the cells with 200 nM calphostin C for 2 h had no effect on the inhibition of Kir2.1 induced by RhoA-QL. The current density was reduced to -16.7 ± 2.3 pA/pF (n = 5) in cells preincubated with calphostin and were -17.8 ± 1.6 pA/pF (n = 18) in control RhoA-QL-transfected cells.

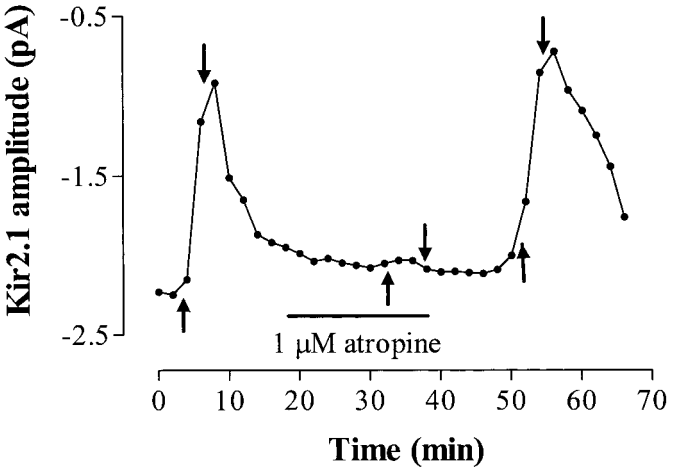

Fig. 2. Time course of inhibition of Kir2.1 by the m1 muscarinic receptor. Current amplitudes were measured at the end of the 200-ms step from -60 to -120 mV by averaging the amplitudes of the last 5 ms and plotted against time. Carbachol (50 μM) was applied at the up arrows and washed off at the down arrows. Incubation with 1 μM atropine inhibited the response to carbachol in a reversible manner.

Involvement of Various Rho Effectors and Kinases. Rho has been shown to activate several proteins including the serine-threonine kinase, Rho kinase (ROK), and phosphatidylinositol 3-kinase [PI(3)K] (see Sah et al., 2000), which phosphorylates phopshatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$] to form phosphatidylinositol-3,4,5-trisphophate. Thus, we examined the effects of inhibitors of these kinases on the muscarinic receptor-induced inhibition of Kir2.1. Preincubation with 10 μM Y-27632, an inhibitor of ROK, for 30 to 45 min had no effect on Kir2.1 current amplitude or on the response to muscarinic receptor stimulation (Fig. 4). However, this treatment with Y-27632 was effective in inhibiting ROK, inasmuch as serum-induced cell cortical retraction was inhibited in serum-starved cells that were preincubated with 10 μM Y-27632 (data not shown), as demonstrated previously by Maddox and Burridge (2003). Briefly, tsA cells were maintained in serum-free medium for 24 h, followed by a 45-min preincubation with Y-27632 or vehicle. The cells were then placed in serum-containing growth medium, and the morphology of the cells was observed 2 h later. Wortmannin, an inhibitor of PI(3)K, was also without effect on Kir2.1 amplitudes or on muscarinic receptor-induced inhibition when preincubated at a concentration of 100 nM for 30 to 45 min (Fig. 4). This treatment with wortmannin was effective in inhibiting PI(3)K, because wortmannin at 100 nM reduced the immunohistochemical labeling of the early endosomal autoantigen 1 (data not shown), as noted previously in human embryonic kidney 293 cells by Hunyady et al. (2002).

Recently, Kir2.1 was shown to be inhibited by constitutively activated Ras, via a mechanism involving the MAPK pathway (Giovannardi et al., 2002). This effect was completely abolished by the mitogen-activated protein kinase kinase (MEK) inhibitor PD98059. Thus, to determine whether MAPKs were involved in the muscarinic receptor-induced inhibition of Kir2.1, the cells were preincubated with 40 μM PD98059 for 2 h before recording as used in the study

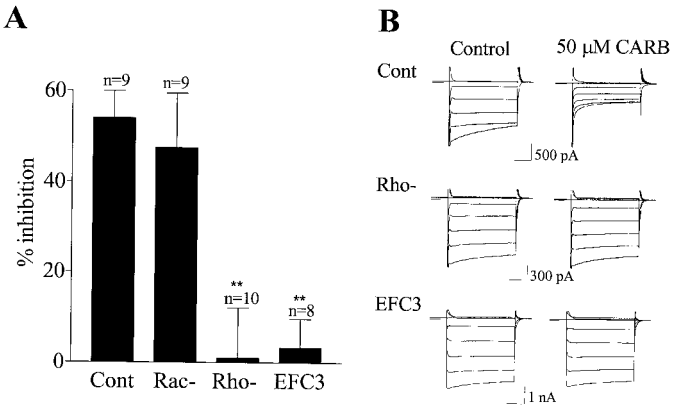

Fig. 3. Inhibition of Rho abolished the m1 muscarinic receptor-induced inhibition of Kir2.1. A, in cells cotransfected with Kir2.1, m1, GFP, and either the neomycin resistance gene (Cont) or the dominant-negative mutant of Rac 1, *N*-17-Rac1 (Rac$-$), application of 50 μM carbachol induced a robust reduction in Kir2.1 current amplitude. In cells cotransfected with Kir2.1, m1, GFP, and either the dominant-negative mutant of Rho, N19-RhoA (Rho$-$) or the plasmid encoding the C3-transferase toxin (EFC3), application of 50 μM carbachol was without effect on the Kir2.1 current amplitude. B, the superimposed currents were elicited by 200-ms steps from -60 mV to potentials ranging from -160 to -20 mV in 20-mV increments. Left, representative currents under control conditions; right, currents recorded during application of 50 μM carbachol from a flow pipette (50 μM CARB). Horizontal bars represent 50 ms. $\star\star$, $p < 0.01$.

by Giovannardi et al. (2002). As shown in Fig. 4, PD98059 had no effect on the m1 muscarinic receptor-induced inhibition of Kir2.1, suggesting that MAPKs are not involved in the mechanism. The effectiveness of this treatment with PD98059 was assessed by determination of insulin-like growth factor 1 (IGF-1)-induced cell proliferation using the methods of Du et al. (2003). Briefly, tsA cells were incubated in serum-free medium for 24 h. The cells were then stimulated with 100 ng/ml IGF-1 to induce proliferation. Cell numbers counted 24 h later indicated that preincubation with 40 μM PD98059 for 2 h reduced the cellular proliferation induced by IGF-1 to nonstimulated levels (data not shown).

The above data suggest that the inhibition of Kir2.1 via Rho is probably not mediated through a mechanism involving phosphorylation by these particular kinases.

Discussion

The purpose of the present study was to elucidate the role of the small GTPases in mediation of the inhibition of Kir2.1 by G-protein-coupled receptors. To achieve this goal, the effects of dominant-negative mutant GTPases and C3 exoenzyme, a specific inhibitor of Rho, were tested on the m1 muscarinic receptor-induced inhibition of Kir2.1. A marked attenuation of the inhibitory response was observed in cells transfected with the dominant-negative mutant of Rho or with the C3 toxin. This indicated that Rho is required for mediation of the inhibition of Kir2.1. This was further confirmed by use of the activated mutant of Rho, which induced an inhibition of Kir2.1. The mechanism by which Rho induces inhibition of Kir2.1 is unclear at this time. However, there are several potential mechanisms, as discussed below.

Rho has been implicated in multiple effects, including formation of stress fibers and regulation of the actin cytoskeleton as well as regulation of cell adhesion, phospholipase D, endocytosis, Na/H exchange, and intracellular pH (Sah et al., 2000; Etienne-Manneville and Hall, 2002; Schmidt and Hall, 2002). Therefore, Rho may be involved in altering channel membrane targeting and recycling. Tong et al. (2001) noted that tyrosine 242 of Kir2.1 was involved in activation of endocytosis of the channel, and Giovannardi et al. (2002) have shown that MAPK activation stimulated removal of Kir2.1 from the membrane. However, it would seem that MAPK activation is not involved in mediation of the muscarinic receptor-induced inhibition of Kir2.1 because of the lack of effect noted with the MEK inhibitor PD98059.

The cascade of proteins downstream of activation of Rho and potentially involved in mediation of many of these functions has not yet been fully elucidated. However, several proteins have been shown to selectively couple with the activated GTP-bound form of Rho, including two serine/threonine protein kinases, PKN and ROK (Sah et al., 2000). Rho kinase in turn inhibits the activity of myosin light chain phosphatase. Activation of a volume-activated anion channel has been shown to be transduced by Rho via Rho kinase and myosin light chain kinase (Eggermont et al., 2001). In the present study, use of the Rho kinase inhibitor Y-27632, at concentrations similar to those used by Nilius et al. (1999) to

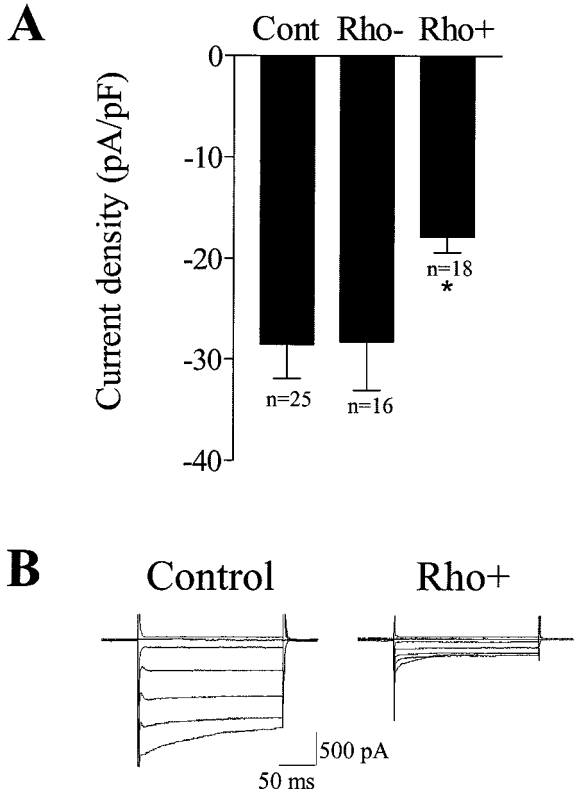

Fig. 4. The activated mutant of Rho inhibited Kir2.1. Cotransfection with Kir2.1, m1, GFP, and either neomycin resistance (Cont) or the dominant-negative mutant of Rho, N19-RhoA (Rho−), produced robust inwardly rectifying potassium currents. In cells cotransfected with Kir2.1, m1, GFP, and the activated mutant of Rho, RhoA-QL (Rho+), Kir2.1 current amplitude was reduced in comparison to control currents. A, current amplitudes were normalized to current density to account for differences in cell size. ⋆, statistically significantly different from control using the Mann-Whitney U test, $p < 0.05$. B, the superimposed current traces were elicited by 200-ms steps from −60 mV to potentials ranging from −160 to −40 mV in 20-mV increments. Left, representative currents under control conditions; right, currents recorded from cells transfected with RhoA-QL (Rho+).

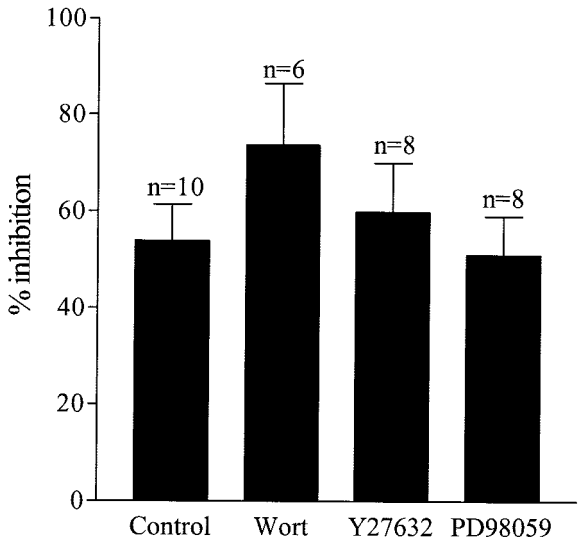

Fig. 5. Inhibition of various protein kinases had no effect on the m1 muscarinic receptor-induced inhibition of Kir 2.1. Application of 50 μM carbachol induced a robust reduction in Kir2.1 current amplitude in cells cotransfected with Kir 2.1, m1, and GFP under control conditions (Control) and after incubation for 30 to 45 min with the PI(3)K inhibitor wortmannin (Wort; 100 nM). Preincubation with the ROK inhibitor Y-27632 (10 μM) for 30 to 45 min or for 2 h with 40 μM PD98059, a MEK inhibitor, was also without effect on the carbachol-induced inhibition of Kir2.1.

inhibit ROK, had no effect on the muscarinic receptor-induced inhibition of Kir2.1, and thus it is unlikely that ROK mediates the response. In addition, Rho GTPases have been shown to activate PI(3)K (reviewed by Sah et al., 2000), which phosphorylates $PI(4,5)P_2$ to form phosphatidylinositol-3,4,5-trisphosphate. Phosphatidylinositol-3,4,5-trisphosphate serves as a trigger for numerous downstream events, such as actin rearrangement, cell growth, cell polarity, and cell survival. Storey et al. (2002) showed that PI(3)K was involved in the Rac-induced attenuation of *ether-a-go-go*-related potassium channels by inhibition of PI(3)K with wortmannin. A similar mechanism does not seem to be involved in the m1 receptor-induced inhibition of Kir2.1, because wortmannin up to 100 nM was without effect. At this concentration, wortmannin is thought to be specific for inhibition of PI(3)K, although at slightly higher concentrations, wortmannin has been shown to inhibit MAPK, myosin light chain kinase, and phosphatidylinositol 4-kinase.

Rho has also been shown to activate phosphatidylinositol 4-phosphate 5-kinase (see Sah et al., 2000), the enzyme that catalyzes the formation of $PI(4,5)P_2$, and thus, it is believed that Rho is a critical regulator of the cellular pool of $PI(4,5)P_2$. $PI(4,5)P_2$ has been shown to be necessary for Kir 2.1 channel function (Huang et al., 1998; Rohacs et al., 1999). Therefore, it is possible that the effects of Rho on Kir2.1 are in some way mediated through control of $PI(4,5)P_2$ levels.

The cascade of proteins upstream of Rho is currently unknown and may involve the heterotrimeric G-protein, Gq (see Kjoller and Hall, 1999, for review). We have previously shown that Gq enhances the muscarinic receptor-induced inhibition of Kir2.1 (Firth and Jones, 2001). Recently, Rho has been shown to be activated by the m1 muscarinic receptor via a mechanism involving Gq (Chikumi et al., 2002). However, although the m1 muscarinic receptor increased activation of Rho via Gq, it may not be the only effect, because Sagi et al. (2001) noted that stimulation of Gq also enhanced signals downstream of Rho. A link between the heterotrimeric G-proteins G12 and G13 and Rho has also been demonstrated. p115 RhoGEF, a GEF or activator of Rho, has been shown to act as a GTPase-activating protein for both of these G-proteins (see Sah et al., 2000). Another putative RhoGEF was recently identified, PDZ-RhoGEF (see Sah et al., 2000). Also, it is thought that PKC may function as a GTPase-activating protein for Gq and a GEF for Rho (see Sah et al., 2000). In addition, Rho has been shown to activate PKC. We have shown previously that PKC is also involved in the signal transduction pathway for inhibition of Kir2.1, by the m1 muscarinic receptor, using phorbol esters and the PKC inhibitor, calphostin C (Jones, 1996, 1997). In this study, the inhibition of Kir2.1 by PMA was abolished by the C3 toxin, suggesting that PKC either acts upstream of Rho, or both pathways are required to be activated. However, because the inhibition of Kir2.1 by activated RhoA was not affected by the PKC inhibitor, calphostin C, it would seem that PKC acts upstream of Rho.

The inhibition of Kir2.1 by a mechanism involving Rho may have profound effects both under normal and pathological conditions. For example, in the heart, IK1, the inwardly rectifying potassium conductance expressed in the ventricles is reduced in hypertrophy (Tomaselli and Marban 1999; Lopatin and Nichols, 2001). In addition, reductions in Kir 2.1 underlying IK1 resulted in prolonged action potential durations and a destabilization of the membrane. Furthermore, a mutation in Kir2.1 resulting in loss of function has been shown to cause Andersen's syndrome, a disease characterized by cardiac arrhythmias and periodic paralysis (Plaster et al., 2001). Chronic activation of Rho has been shown to lead to development of hypertrophy (Sah et al., 2000); thus, Rho activation may play an important role in inhibition of Kir2.1, potentially resulting in ventricular arrhythmias. Kir2.1 is also a pivotal channel in regulation of vascular smooth muscle tone (Zaritsky et al., 2000), where Rho has also been shown to provide a modulatory influence (Sah et al., 2000). In fact, many cellular events have been shown to involve the small GTPases and Rho in particular. Thus, the participation of Rho in ion channel regulation is probably a widespread phenomenon both in the peripheral and central nervous system as well as in all excitable cells.

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