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Modulation of the Inwardly Rectifying Potassium Channel IRK1 by the m1 Muscarinic Receptor

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SUMMARY

Modulation of the inwardly rectifying potassium channel (IRK1) by the m1 muscarinic receptor was studied with the whole-cell patch-clamp recording technique with the use of a mammalian expression system. After transfection with IRK1 and m1 muscarinic receptor genes, tsA cells expressed a cesium-sensitive inwardly rectifying potassium conductance that was reduced on application of the muscarinic receptor agonist carbachol. This reduction was reversible on washout of carbachol and could be completely inhibited by the muscarinic receptor antagonist atropine. Conversely, stimulation of the m2 muscarinic receptor, when coexpressed with IRK1, resulted in no change in IRK1 current amplitude. Phorbol-12,13-dibutyrate, an activator of protein kinase C (PKC), mimicked the effect of m1 muscarinic receptor stimulation by inhibiting the IRK1 conductance. Preincubation with staurosporine or the specific PKC inhibitor calphostin C, before application of carbachol, fully prevented the inhibition of IRK1 by m1 muscarinic receptor stimulation. Administration of 8-bromo-cAMP, an activator of protein kinase A, and thapsigargin, a stimulator of intracellular calcium release, had no effect on IRK1, suggesting that these second messengers were not involved in the m1 muscarinic receptor-induced response. Therefore, the data indicate that the m1 muscarinic receptor inhibits IRK1, presumably via stimulation of PKC. As IRK1 is widely distributed throughout the central nervous system, it is possible that such an action on IRK1 underlies the inhibitory effects of muscarinic receptor stimulation on inwardly rectifying potassium conductances observed in the brain.

Inwardly rectifying potassium channels play a major role in maintaining the membrane potential of cells near the potassium equilibrium potential and in permitting long depolarizations in excitable cells due to the inward rectification at depolarized potentials (1). Therefore, modulation of these channels will have a profound effect on the excitability of the cell. Modulation of inwardly rectifying potassium conductances by muscarinic receptors has been the focus of many studies. In particular, the stimulation of an inward rectifier ($I_{K.ACh}$) by the m2 muscarinic receptor in heart has been studied in detail (for reviews, see Refs. 1–6). This action is believed to be due to a direct effect of heterotrimeric G protein $\beta\gamma$ subunits acting on the channel (7–9). However, muscarinic receptor activation has also been observed to cause inhibition of inwardly rectifying potassium conductances in central neurons (10, 11).

In 1993, the first mammalian inwardly rectifying potassium channels ROMK1 (12) and IRK1 (13) were cloned. IRK1 was expression cloned in *Xenopus* oocytes from RNA derived from mouse macrophages. This channel has been shown to be present throughout the nervous system and is abundant in both heart and brain (13, 14). However, IRK1 was shown not to be modulated by GTPγS and thus it was thought unlikely to represent the muscarinic receptor-stimulated potassium channel $I_{K.ACh}$ in heart (13). Another inwardly rectifying potassium channel cloned from heart, known as GIRK1 (15) or KGA (16), was subsequently shown to be the channel that could be activated by the m2 muscarinic receptor.

As GIRK1 requires muscarinic receptor stimulation to be active, it is unlikely that GIRK1 represents the inwardly rectifying potassium channel inhibited by muscarinic receptors. On the other hand, IRK1 is normally active under control conditions and therefore is a likely candidate for this effect. In the current study, I investigated the effects of muscarinic receptor stimulation on IRK1.

There are five different subtypes of muscarinic receptor (m1–m5) (for reviews, see Refs. 17–20). m2 and m4 activate inwardly rectifying potassium conductances (15, 16, 21) and inhibit cAMP formation. m1, m3, and m5 have been shown to activate phospholipase C, resulting in formation of $InsP_3$ and DAG. DAG stimulates PKC activity, whereas $InsP_3$ triggers

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ABBREVIATIONS: IRK, inwardly rectifying K^+ channel; $InsP_3$, inositol trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; PKA, protein kinase A; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PDBu, phorbol-12,13-dibutyrate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $I_{K.Ach}$, muscarinic receptor-activated, inwardly rectifying potassium channel.

release of calcium from intracellular stores. Arachidonic acid, a metabolite of DAG, is also released on stimulation of these muscarinic receptors. m1, m3, and m4 have been shown to stimulate cAMP formation in some cells (22, 23). The m1 muscarinic receptor was therefore chosen for study as a representative receptor capable of activating a wide variety of second messengers.

Materials and Methods

Transfection of tsA cells. tsA cells were grown in 35-mm dishes at 37° in 5% CO_2 and in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The cells were transiently transfected with 10 μg of human m1 or m2 and 10 μg of mouse IRK1 plasmid DNA by the calcium phosphate precipitation method (24). m1 and m2 were inserted into the vector pcD as reported previously (25). IRK1 was inserted into the vector pcDNA1 (Invitrogen) as reported previously (13). The cells were recorded from 2–3 days after transfection.

Muscarinic receptor binding assay. Cells were harvested, resuspended, and homogenized in 25 mM sodium phosphate buffer with 5 mM $MgCl_2$. Binding assays were initiated by the addition of 800 μl of membranes to 50 μl of atropine or buffer and 50 μl of [^{3}H]*N*-methylscopolamine (85 Ci/mmol, DuPont-NEN). The membranes were incubated for 1 hr at room temperature. Nonspecific binding was determined with 1 μM atropine.

Electrophysiology. The whole-cell patch-clamp recording technique was performed at room temperature (21–23°) with a LIST EPC-7 patch-clamp amplifier. Cells were visualized with the use of an inverted phase contrast microscope (AXIOVERT 100) at a magnification of 200×. Patch electrodes were pulled from thin wall borosilicate glass capillary tubing on a three-stage horizontal puller (Mecanex BB.CH.PC, Basel, Switzerland) and had resistances of ~5 MΩ. Currents were filtered at 1 KHz, digitized through a DMA interface analog/digital converter (MIO, National Instruments, Austin, TX), and sampled at 2 KHz by a Macintosh Quadra 800 computer with the use of Axodata software (Axon Instruments, Foster City, CA).

Cells were voltage-clamped at a holding potential of −60 mV, and the currents evoked by a series of depolarizing steps were recorded. Current-voltage relationships were constructed from the currents measured at the end of 200-msec steps from −160 mV to 50 mV in 10-mV increments with the use of Axograph (Axon Instruments). Carbachol was applied with a low pressure ejection system from a micropipette placed close to the cell. Drugs were applied directly to the bath and were washed out by perfusion of extracellular solution (2 ml/min). Results are expressed as mean ± standard error.

Solutions and drugs. The extracellular recording solution consisted of 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, and 20 mM glucose, pH adjusted to 7.4 with 1 M NaOH. The osmolarity of the solution was adjusted to 325–330 mmol/kg. The intracellular patch pipette solution contained 150 mM K-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, pH adjusted to 7.2 and osmolarity adjusted to 315–320 mmol/kg.

PDBu, carbachol, staurosporine, and atropine were obtained from Sigma Chemical Co. (St. Louis, MO); 1,2-dioctanoylglycerol from Molecular Probes (Eugene, OR); and calphostin C and thapsigargin from RBI (Natick, MA).

Results

IRK1 and muscarinic receptor expression in tsA cells. The modulation of the mouse inwardly rectifying potassium channel IRK1 by the human m1 and m2 muscarinic receptors was studied by expression of the proteins in the human embryonic kidney cell line tsA 201. The tsA cells had been modified by stable transfection with a simian virus 40 temperature-sensitive T-antigen as described previously (26). Before transfection, tsA cells showed little inward current and displayed a large outward, TEA-sensitive potassium conductance (Fig. 1A). This conductance had the slow activation rate characteristic of the delayed rectifier-type potassium channel (Fig. 1B). Application of carbachol to control cells transfected with m1 muscarinic receptor alone resulted in a reduction of outward current but no change in the leak conductance measured from −130 to −70 mV (Fig. 1C). Muscarinic receptor expression was confirmed by [^{3}H]*N*-methylscopolamine binding to m1- and m2-transfected cells. Binding sites were increased from 4.4 ± 0.3 (six experiments) fmol/mg protein before transfection to 835 ± 191 (six experiments) fmol/mg protein 2 days after transient transfection with the m1 muscarinic receptor and from 2.9 ± 0.6 (three experiments) fmol/mg protein before transfection to 605 ± 24 (three experiments) fmol/mg protein 2 days after transient transfection with the m2 muscarinic receptor. Binding was reduced to control levels in assays incubated with 1 μM atropine (nine experiments), indicating successful high level muscarinic receptor expression.

Transfection of the tsA cells with the inwardly rectifying potassium channel IRK1 resulted in expression of a large inward conductance (Fig. 2) closely resembling currents observed with expression of IRK1 in *Xenopus* oocytes (13). The inward current reversed at −60 ± 3 mV (21 experiments) and was completely inhibited by 1 mM cesium (Fig. 2A). Subtraction of cesium-inhibited currents from control cur-

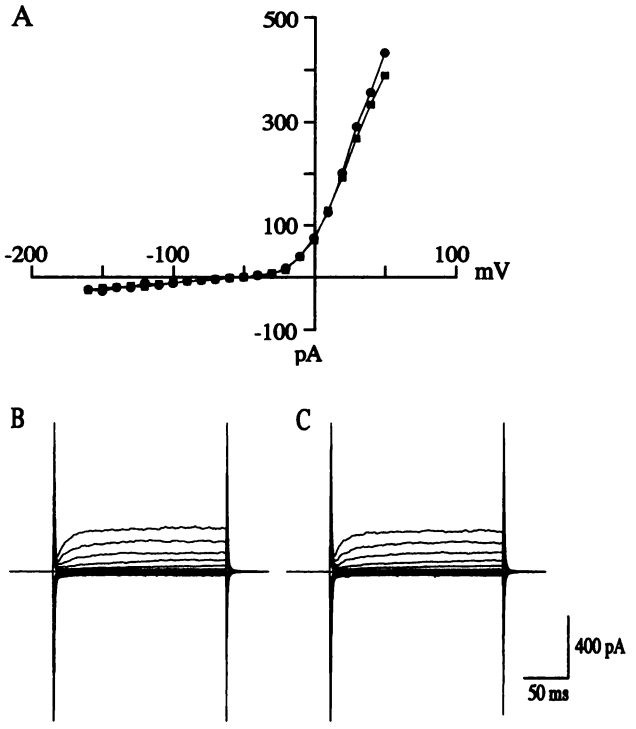

Fig. 1. tsA cells express little inward current before transfection and display an outward delayed rectifier-type potassium conductance. A, Current-voltage curve constructed from measurements taken at the end of 200-msec steps from a holding potential of −60 mV to a variety of test potentials in control (●) and after application of 50 μM carbachol from a pressure ejection pipette (■). B, Superimposed current traces from a cell in control conditions at a holding potential of −60 mV with steps shown from −160 to 50 mV in 10-mV increments. C, Same cell as in A and B after application of 50 μM carbachol.

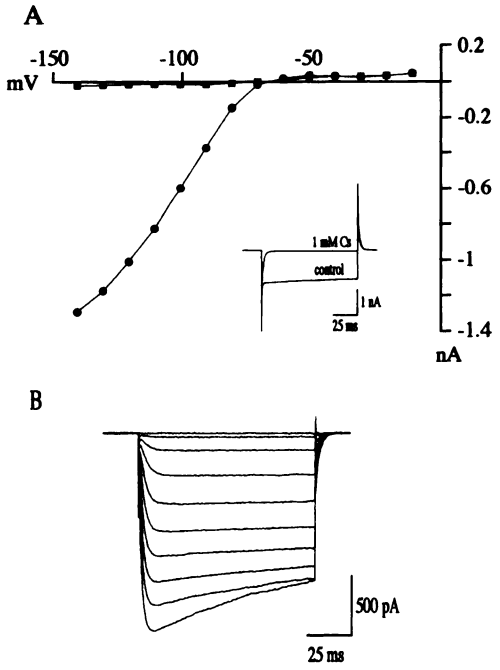

Fig. 2. tsA cells express a large inwardly rectifying potassium conductance after transfection with IRK1. A, Inward current observed after transfection with IRK1 (●) is abolished by 1 mM cesium (■). *Inset*, current traces during a 200-msec step from −60 to −120 mV in control and after cesium application. B, Traces are result of subtraction of traces recorded in the presence of cesium from control recordings of the same cell as in A. Traces are shown from a holding potential of −60 mV with steps from −150 to 0 mV.

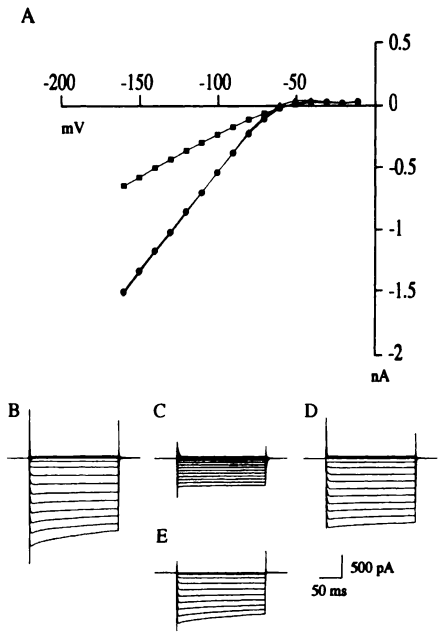

Fig. 3. Muscarinic receptor stimulation results in a reduction of inwardly rectifying current. Transfection of a tsA cell with IRK1 and m1 results in a large inward current shown (A) in control (●), which was reduced after application of 50 μM carbachol (■) and recovers to control values after washout of carbachol (♦). Current values were measured at the end of 200-msec steps from −60 mV. Superimposed current traces from the same cell as in A of voltage steps from −160 mV to −20 mV are shown in control (B) after 50 μM carbachol (C) and after washout (D). E, resultant current traces after subtraction of currents measured in the presence of carbachol from control currents.

rents revealed the inwardly rectifying potassium current (Fig. 2B). IRK1 is a strongly rectifying potassium conductance, and thus there is little outward current passing at potentials more depolarized than E_K, the potassium equilibrium potential.

m1 muscarinic receptor inhibits IRK1. In cells transfected with both IRK1 and m1, stimulation of the muscarinic receptor reduced the inwardly rectifying potassium conductance in a reversible manner, as shown in Fig. 3. The application of 10–100 μM carbachol to the cells via a pressure ejection pipette resulted in a decrease in both inward and outward current amplitude (21 experiments). During a step to −100 mV, current amplitude was reduced from −1.39 ± 0.15 nA (16 experiments) in control to −1.02 ± 0.17 nA (16 experiments) after application of 50 μM carbachol. This indicates an inhibition of 32 ± 7% (16 experiments). As seen in the current-voltage curve constructed from currents measured at the end of the 200-msec steps to a variety of potentials, this reduction in amplitude was echoed with a reduction in conductance (Fig. 3A). Cord conductance, measured in the linear portion of the current-voltage curve from −130 to −70 mV, was reduced from 41.4 ± 5.1 nS to 29.3 ± 5.4 nS (16 experiments) in the presence of 50 μM carbachol. Similarly, the outward portion of the inwardly rectifying potassium conductance, measured at −50 mV, was reduced from 0.21 ± 0.06 nA in control to 0.07 ± 0.04 nA (16 experiments) in the presence of 50 μM carbachol, indicating a 52 ± 7% reduction. The outward currents were measured at −50 mV to avoid contamination by the voltage-dependent delayed rectifier outward potassium conductance, which usually activated between −10 and −20 mV. Subtraction of the carbachol-inhibited current traces from control traces (Fig. 3E) revealed inwardly rectifying potassium currents that closely resembled those of the cesium-subtracted currents.

The reduction in current amplitude induced by application of carbachol was fully reversed on washout (Figs. 3D and 4). Control currents of −1.60 ± 0.24 nA (nine experiments) were reduced to −1.15 ± 0.26 nA (nine experiments) in response to carbachol application and returned to control values of −1.58 ± 0.27 nA (nine experiments) after washout.

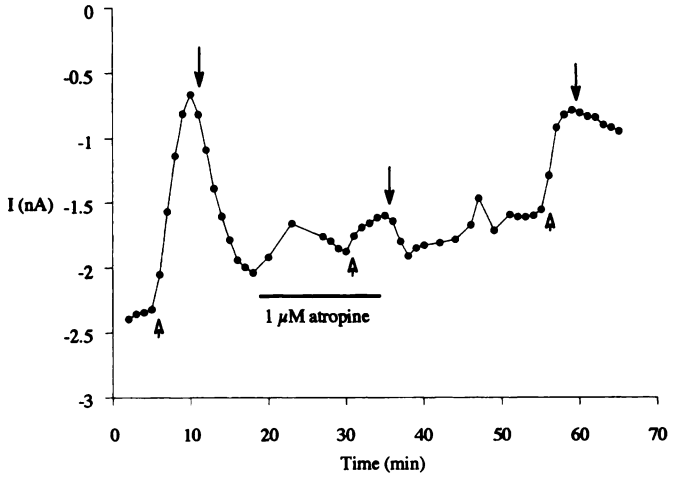

Fig. 4. Carbachol-induced inhibition of IRK1 is blocked by muscarinic antagonist atropine. Shown is current amplitude measured in a cell transfected with m1 and IRK1 at the end of 200-msec steps from −60 to −160 mV. *Open arrows*, application of 50 μM carbachol (30-sec application). *Solid arrows*, start of a 5-min washout period.

The time course of muscarinic receptor-induced inhibition of IRK1 current amplitude is shown in Fig. 4A, revealing that the carbachol effect slowly reaches a peak, which is reversed only on washout of the carbachol. The response to carbachol was inhibited by incubation with 1 μM atropine for 10 min. Carbachol induced a 50 ± 9% (five experiments) decrease in inward current amplitude measured at the end of a step to −100 mV before the addition of atropine and reduced currents by only 19 ± 7% (five experiments) in the presence of the atropine.

For purposes of comparison, the effects of stimulation of the m2 muscarinic receptor on the inward rectifier were studied in cells cotransfected with m2 and IRK1 cDNA. Application of carbachol had no effect on IRK1 current amplitude, being 0.72 ± 0.16 nA (eight experiments) in control and 0.73 ± 0.17 nA (eight experiments) after application of 100 μM carbachol. This suggests that the m1 muscarinic receptor-induced inhibition of IRK1 was specific to the m1 muscarinic receptor.

Role of second messengers in mediating the muscarinic effects. Due to the slow nature of the carbachol response, indicating the possibility of mediation by a second messenger, and because the m1 muscarinic receptor activates a variety of second messengers, several types of second messenger were tested for their effects on IRK1. Uchimura and North (10) demonstrated that the muscarine-induced inhibition of an inwardly rectifying potassium conductance in neurons of the nucleus accumbens could be mimicked by application of a phorbol ester; therefore, PDBu, a phorbol ester that stimulates PKC, was initially tested.

The application of PDBu (100 nM to 2 μM) produced a reduction in IRK1 conductance and current amplitude (six experiments) (Fig. 5A). These effects seem to be similar to those induced by carbachol. PDBu (1 μM) reduced current amplitude by 67 ± 10% (three experiments). The application of 10 μM 1,2-dioctanoylglycerol, an analogue of diacylglycerol that also stimulates PKC, also reduced IRK1 current amplitude (two experiments).

To determine whether the effect of carbachol on IRK1 involved stimulation of PKC, the cells were exposed to staurosporine, an inhibitor of PKC (although not highly selective for PKC), and to calphostin C, a more selective inhibitor of PKC (27, 28). In the presence of 500 nM staurosporine, carbachol had no effect on IRK1 in m1 muscarinic receptor-transfected cells. Current amplitudes measured at the end of the 200-msec step to −100 mV were 0.42 ± 0.09 nA (12 experiments) before and 0.48 ± 0.16 nA (12 experiments) after the application of 100 μM carbachol. Preincubation of the cells with 200 nM calphostin C for 40 min before electrophysiological recordings resulted in an inhibition of the effect of carbachol on IRK1 current amplitude (Fig. 6). Carbachol (50 μM) reduced current amplitude measured during a step to −100 mV by 35 ± 8% (nine experiments). The carbachol-induced effect was reduced to a 3 ± 6% (five experiments) inhibition of current amplitude in the presence of calphostin C. These observations provide evidence for the possible role of PKC in the transduction of the carbachol effect.

Release of intracellular calcium by m1 muscarinic receptor stimulation may also play a role in the inhibition of IRK1. To test this, thapsigargin was used to stimulate release of intracellular calcium. Thapsigargin (200 nM) induced a small inhibition of IRK1 current amplitude. Control currents were

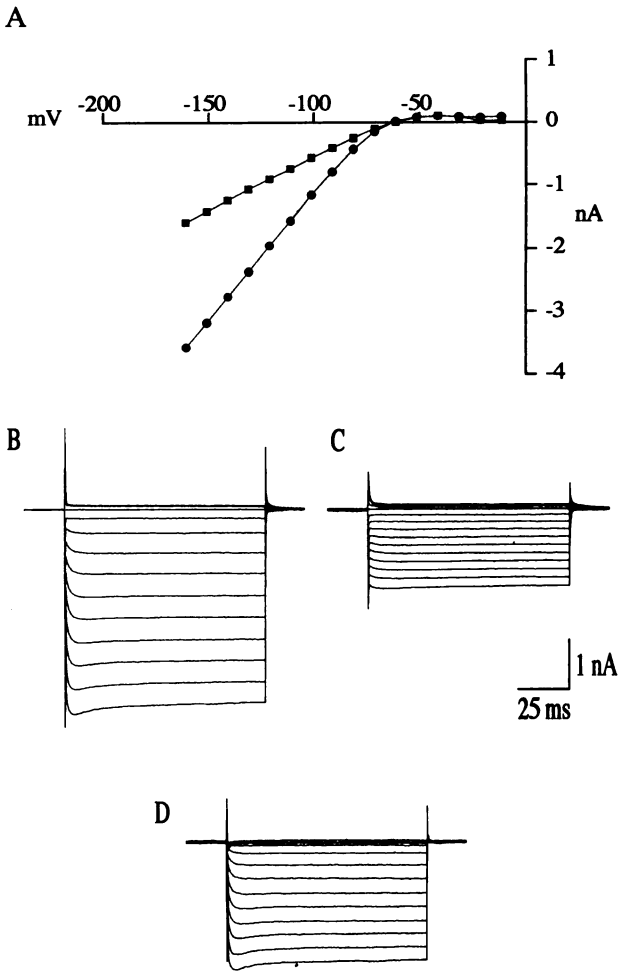

Fig. 5. PDBu inhibits IRK1. A, Application of 500 nM PDBu (■) reduced control inward currents (●) by ~50%. Currents were measured at the end of 200-msec steps from a holding potential of −60 mV. Current traces from this cell are shown in control (B) and after application of PDBu (C). Subtraction of the PDBu-inhibited traces from control revealed the PDBu-inhibited portion of the current shown in D. Current traces are shown of steps from −160 to −20 mV in 10-mV increments.

reduced from −1.50 ± 0.46 nA to −1.26 ± 0.45 nA (five experiments) in the presence of thapsigargin. This reduction, however, was not found to be statistically significant as tested with a paired Student's *t* test.

As the m1 muscarinic receptor has also been shown to increase cAMP levels in cells (22), the effects of 8-bromo-cAMP were examined on the IRK1 currents. 8-Bromo-cAMP (500 μM) did not significantly change the current amplitude at the end of a step to −100 mV, being −1.58 ± 0.30 nA in control and −1.62 ± 0.35 nA in the presence of 8-bromo-cAMP (four experiments).

Discussion

This study demonstrates that IRK1 can be modulated by the m1 muscarinic receptor. The muscarinic inhibition of IRK1 was mimicked by PDBu and abolished by staurosporine and calphostin C. This indicates that inhibition of IRK1 by the m1 muscarinic receptor is probably due to stimulation of PKC. This conclusion is supported by Fakler *et al.* (29), who showed that IRK1 could be inhibited by SC10, a stimulator of PKC. It is possible that the inhibition of IRK1 by the m1

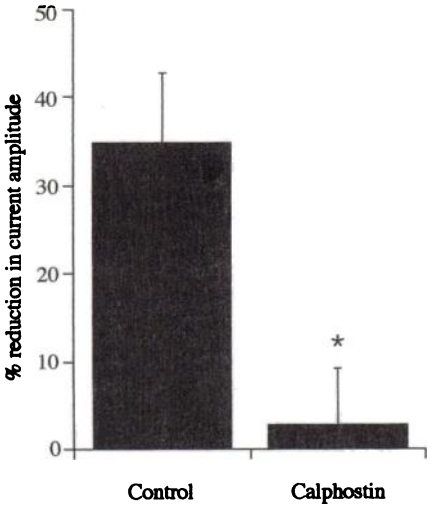

Fig. 6. Calphostin C inhibited muscarinic responses. Carbachol reduced IRK1 currents in m1- and IRK1-transfected cells by ~35% (nine experiments). Preincubation of the cells for 40 min with 200 nM calphostin C inhibited the carbachol-induced reduction in IRK1 current amplitude (five experiments). Current measurements were taken from the end of 200-msec steps from −60 to −100 mV in the presence and absence of 50 μM carbachol. *, Statistically significantly different as determined with Student's *t* test ($p < 0.01$).

muscarinic receptor represents the muscarinic receptor-induced inhibition of inwardly rectifying potassium conductances in the brain (10, 11). Support for this suggestion is provided by the intracellular recording studies from neurons of the nucleus accumbens (10). In these studies, muscarinic receptor stimulation inhibited an inwardly rectifying potassium conductance that was mimicked and blocked by application of an activator of PKC.

The m1 muscarinic receptor stimulates various second messenger pathways, including activation of PLC, which leads to formation of $InsP_3$ and release of intracellular calcium, and DAG, which results in activation of PKC. Raised levels of intracellular calcium concentration have been shown to inhibit inwardly rectifying potassium channels in some studies (30, 31) and to activate inward rectifiers in others (32, 33). In this study, thapsigargin, an agent that releases calcium from endoplasmic reticulum (34), was used to increase intracellular calcium concentrations. Thapsigargin had no significant effect on IRK1 current amplitude, suggesting that IRK1 was not modulated by calcium.

In some cells, the m1 muscarinic receptor has also been shown to increase cAMP concentrations (22), possibly via stimulation of adenylyl cyclase (23). This increase in cAMP levels stimulated by the m1 muscarinic receptor might also contribute to the inhibition of IRK1. However, this mechanism can be ruled out as application of 8-bromo-cAMP to the cells did not significantly affect the IRK1 current. As 8-bromo-cAMP can activate PKA, this result differs from that of Fakler *et al.* (29), who noted an increase in IRK1 current on application of the catalytic subunit of PKA. Because they obtained recordings from inside-out macropatches, I assume that differences in effect were due to differing recording conditions and treatments. However, as PKA caused an increase in IRK1 in their study, it would be unlikely that cAMP was involved in the m1 muscarinic receptor-induced inhibitory effect.

Previous whole-cell recordings of IRK1 have been noted to show considerable rundown over the time course of the experiment. It is assumed that rundown of the channel current amplitude in this study was kept to a minimum due to the inclusion of high concentrations of Mg-ATP in the patch pipette, which has been shown to maintain the current (29). This is also likely to be the explanation for why GTPγS was without effect in the initial studies of Kubo *et al.* (13), as inhibition of IRK1 by GTPγS was probably indistinguishable from rundown.

A family of IRK1-like inwardly rectifying potassium channels has been cloned from a variety of tissues since the initial cloning of IRK1; these include IRK2 (35–37) and IRK3 (38). It will be interesting to determine whether these are also modulated by the muscarinic receptors. There are several PKC consensus sequence sites in each of these IRK subtypes; however, their locations seem to be somewhat different.

Acknowledgments

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References

1. Hille, B. *Ionic Channels in Excitable Membranes*, Ed. 2. Sinauer Associates, Sunderland, MA (1992).
2. Hartzell, H. C. Regulation of cardiac ion channels by catecholamines, acetylcholine and second messenger systems. *Prog. Biophys. Mol. Biol.* **52**:165–247 (1988).
3. Brown, A. M., and L. Birnbaumer. Ionic channels and their regulation by G protein subunits. *Annu. Rev. Physiol.* **52**:197–213 (1990).
4. Szabo, G., and A. S. Otero. G-protein mediated regulation of potassium channels in heart. *Annu. Rev. Physiol.* **52**:293–305 (1990).
5. Clapham, D. E. Direct G protein activation of ion channels. *Annu. Rev. Neurosci.* **17**:144–164 (1994).
6. Yamada, M., A. Terzic, and Y. Kurachi. Regulation of potassium channels by G-protein subunits and arachidonic acid metabolites. *Methods Enzymol.* **238**:394–422 (1994).
7. Logothetis, D. E., Y. Kurachi, J. Galper, E. J. Neer, and D. E. Clapham. The βγ subunits of GTP-binding proteins activate the muscarinic K^+ channel in heart. *Nature (Lond.)* **325**:321–326 (1987).
8. Reuveny, E., P. A. Slesinger, J. Inglese, J. M. Morales, J. A. Iniguez-Lluhi, R. J. Lefkowitz, H. R. Bourne, Y. N. Jan, and L. Y. Jan. Activation of the cloned muscarinic potassium channel by G protein βγ subunits. *Nature (Lond.)* **370**:143–146 (1994).
9. Takao, K., M. Yoshii, A. Kanda, S. Kokubun, and T. Nukada. A region of the muscarinic-gated atrial K^+ channel critical for activation by G protein βγ subunits. *Neuron* **13**:747–755 (1994).
10. Uchimura, N., and R. A. North. Muscarine reduces inwardly rectifying potassium conductance in rat nucleus accumbens neurones. *J. Physiol.* **422**:369–380 (1990).
11. Womble, M. D., and H. C. Moises. Hyperpolarization-activated currents in neurons of the rat basolateral amygdala. *J. Neurophysiol.* **70**:2056–2065 (1993).
12. Ho, K., C. G. Nichols, W. J. Lederer, J. Lytton, P. M. Vassilev, M. V. Kanazirska, and S. C. Hebert. Cloning and expression of an inwardly rectifying ATP-regulated potassium channel. *Nature (Lond.)* **362**:31–38 (1993).
13. Kubo, Y., T. J. Baldwin, Y. N. Jan, and L. Y. Jan. Primary structure and functional expression of a mouse inward rectifier potassium channel. *Nature (Lond.)* **362**:127–133 (1993).
14. Morishige, K.-I., N. Takahashi, A. Jahangir, M. Yamada, H. Koyama, J. S. Zanelli, and Y. Kurachi. Molecular cloning, functional expression and localization of an inward rectifier potassium channel in the mouse brain. *FEBS Lett.* **336**:375–380 (1993).
15. Kubo, Y., E. Reuveny, P. A. Slesinger, Y. N. Jan, and L. Y. Jan. Primary structure and functional expression of a rat G-protein-coupled muscarinic potassium channel. *Nature (Lond.)* **364**:802–806 (1993).
16. Dascal, N., W. Schreibmayer, N. F. Lim, W. Wang, C. Chavkin, L. DiMagno, C. Labarca, B. L. Kieffer, C. Gaveriaux-Ruff, D. Trollinger, H. A. Lester, and N. Davidson. Atrial G protein-activated K^+ channel: expression cloning and molecular properties. *Proc. Natl. Acad. Sci. USA* **90**:10235–10239 (1993).
17. Jones, S. V. P., A. I. Levey, D. M. Weiner, J. Ellis, E. Novotny, S.-H. Yu, F. Dorje, J. Wess, and M. R. Brann. Muscarinic acetylcholine receptors, in *Molecular Biology of Receptors which Couple to G-Proteins* (M. R. Brann, ed.). Birkhauser, Boston, 170–197 (1992).

18. Brann, M. R., H. Jørgensen, E. Burstein, T. Spalding, J. Ellis, S. V. P. Jones, and D. Hill-Eubanks. Studies of the pharmacology, localization and structure of muscarinic acetylcholine receptors. *Ann. N. Y. Acad. Sci.* **707**:225–236 (1993).
19. Caulfield, M. P. Muscarinic receptors: characterization, coupling and function. *Pharmacol. Ther.* **58**:319–379 (1993).
20. Jones, S. V. P. Muscarinic receptor subtypes: modulation of ion channels. *Life Sci.* **52**:457–464 (1993).
21. Jones, S. V. P. m4 muscarinic receptor subtype activates an inwardly rectifying potassium conductance in AtT-20 cells. *Neurosci. Lett.* **147**:125–130 (1992).
22. Jones, S .V. P., C. J. Heilman, and M. R. Brann. Functional responses of cloned muscarinic receptors expressed in CHO-K1 cells. *Mol. Pharmacol.* **40**:242–247 (1991).
23. Dittman, A. H., J. P. Weber, T. R. Hinds, E.-J. Choi, J. C. Migeon, N. M. Nathanson, and D. R. Storm. Novel mechanism for coupling of m4 muscarinic acetylcholine receptors to calmodulin-sensitive adenylyl cyclases: crossover from G protein-coupled inhibition to stimulation. *Biochemistry* **33**:943–951 (1994).
24. Chen, C., and H. Okayama. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**:2745–2750 (1987).
25. Bonner, T. I., N. J. Buckley, A. C. Young, and M. R. Brann. Identification of a family of muscarinic acetylcholine receptor genes. *Science (Washington D. C.)* **237**:527–532 (1987).
26. Chahine, M., P. B. Bennett, A. L. George Jr., and R. Horn. Functional expression and properties of the human skeletal muscle sodium channel. *Pfluegers Arch.* **427**:136–142 (1994).
27. Kobayashi, E., H. Nakano, M. Morimoto, and T. Tamaoki. Calphostin C (UNCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem. Biophys. Res. Commun.* **159**: 548–553 (1989).
28. Bruns, R. F., F. D. Miller, R. L. Merriman, J. J. Howbert, W. F. Heath, E. Kobayashi, I. Takahashi, T. Tamaoki, and H. Nakano. Inhibition of protein kinase C by calphostin C is light dependent. *Biochem. Biophys. Res. Commun.* **176**:288–293 (1991).
29. Fakler, B., U. Brandle, E. Glowatzki, H.-P. Zenner, and J. P. Ruppersberg. Kir 2.1 inward rectifier K^+ channels are regulated independently by protein kinases and ATP hydrolysis. *Neuron* **13**:1413–1420 (1994).
30. Kramer, R. H., and I. B. Levitan. Calcium-dependent inactivation of a potassium current in the *Aplysia* neuron R15. *J. Neurosci.* **8**:1796–1803 (1988).
31. Mukai, M., I. Kyogoku, and M. Kuno. Calcium-dependent inactivation of inwardly rectifying potassium channel in a tumor mast cell line. *Am. J. Physiol.* **262**:C84–C90 (1992).
32. Welsh, M. J., and J. D. McCann. Intracellular calcium regulates basolateral potassium channels in a chloride-secreting epithelium. *Proc. Natl. Acad. Sci. USA* **82**:8823–8826 (1985).
33. Tabcharani, J. A., A. Boucher, J. W. L. Eng, and J. W. Hanrahan. Regulation of an inwardly rectifying K channel in the T84 epithelial cell line by calcium, nucleotides and kinases. *J. Membr. Biol.* **142**:255–266 (1994).
34. Lytton, J., M. Westlin, and M. R. Hanley. Thapsigargin inhibits the sarcoplasmic and endoplasmic reticulum Ca-ATPase family of calcium pumps. *J. Biol. Chem.* **266**:17067–17071 (1992).
35. Koyama, H., K.-I. Morishige, N. Takahashi, J. S. Zanelli, D. N. Fass, and Y. Kurachi. Molecular cloning, functional expression and localization of a novel inward rectifier potassium channel in the rat brain. *FEBS Lett.* **341**:303–307 (1994).
36. Takahashi, N., K.-I. Morishige, A. Jahangir, M. Yamada, I. Findlay, H. Koyama, and Y. Kurachi. Molecular cloning and functional expression of cDNA encoding a second class of inward rectifier potassium channels in the mouse brain. *J. Biol. Chem.* **37**:23274–23279 (1994).
37. Tang, W., and X.-C. Yang. Cloning a novel human inward rectifier potassium channel and its functional expression in *Xenopus* oocytes. *FEBS Lett.* **348**:239–243 (1994).
38. Morishige, K.-I., N. Takahashi, A. Jahangir, M. Yamada, H. Koyama, J. S. Zanelli, and Y. Kurachi. Molecular cloning and functional expression of a novel brain-specific inward rectifier potassium channel. *FEBS Lett.* **346**: 251–256 (1994).

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