Tissue-specific Regulation of G-protein-coupled Inwardly Rectifying K⁺ Channel Expression by Muscarinic Receptor Activation in Ovo*

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We investigated the effects of muscarinic acetylcholine receptor stimulation on the expression levels of the G-protein-coupled inwardly rectifying K⁺ channel (GIRK) subunits using solution hybridization and immunoblot analyses. We report here that treatment of chick embryos in ovo with muscarinic agonists causes decreases in mRNA levels encoding GIRK1 and GIRK4 in atria but does not alter GIRK1 expression in ventricles. In addition, GIRK1 protein levels also demonstrate a decrease in atria upon muscarinic acetylcholine receptor stimulation. Numerous receptors couple to the activation of the GIRK family of inwardly rectifying K⁺ channels; thus, these decreases represent a novel mechanism for regulating physiological responses to chronic agonist exposure.

Muscarinic acetylcholine receptors (mAChRs)1 couple to heterotrimeric G-proteins to regulate multiple effector molecules such as the GIRK family of inwardly rectifying K⁺ channels and the enzymes adenyl cyclase and phospholipase C. Acetylcholine released from parasympathetic neurons binds to mAChRs in the heart, resulting in a negative chronotropic response. There are five subtypes of mAChR, which are the products of different genes; the decrease in heart rate is caused by the activation of m2 subtype of mAChR in mammalian heart and both m2 and m4 subtypes in chick heart (1–3). The negative chronotropic response is due in part to the activation of I_{KACH}, an outward K⁺ current that hyperpolarizes the cell in the sinus node of the atrium. I_{KACH} is activated directly by pertussis toxin-sensitive heterotrimeric G-proteins coupled to m2 and m4 muscarinic receptors without the mediation of second messengers (4). I_{KACH} is due to an assembly of G-protein-coupled inwardly rectifying K⁺ channels comprising two subunits, GIRK1 and GIRK4 (5).

The number of mAChRs expressed in cardiac cells can be regulated by the continued presence of agonist. Persistent activation of cardiac mAChRs leads to receptor sequestration (6), reduction in total receptor number (7), and decreased transcription of the mAChR genes (8). Sequestration of mAChRs occurs within seconds to minutes and involves internalization of receptors from the cell surface (9). Prolonged agonist exposure (hours) results in a decrease in mAChR number and recovery requires de novo protein synthesis (10–12). Another consequence of continued mAChR activation is a decrease in transcription of mAChR genes. In chick heart, both m2 and m4 mAChR mRNA are decreased in a time- and dose-dependent manner (8). This regulation of mAChR mRNA is dependent on both the activation of phospholipase C and the inhibition of adenyl cyclase (13). These decreases in receptors at the cell surface and in gene expression result in a reduced physiological response to subsequent receptor stimulation.

Although many G-protein-coupled receptors exhibit agonist-induced decreases in expression, little information is available on the consequences of receptor stimulation on effector expression. In this study, we determined the effects of mAChR activation in vivo on the expression of GIRK1 and GIRK4 mRNA and GIRK1 protein in chick atria and ventricles.

EXPERIMENTAL PROCEDURES

Materials—Atropine sulfate and carbamylcholine chloride (carbachol) were obtained from Sigma. Chick embryos were obtained from H&E International (Redmond, WA) and maintained at 38 °C in a humidified incubator. Embryos were 9 days old at the time of dissection. Administration of Drugs in Ovo—Drugs for administration in ovo were delivered as described in Halvorsen and Nathanson (7). Briefly, a small opening in the shell was made, and drugs dissolved in PBS (0.8% NaCl, 0.02% KCl, 0.12% Na₂HPO₄, 0.02% KH₂O₄, 0.01% MgCl₂, 0.01% CaCl₂, pH 7.4) were carefully layered onto the inner embryonic membrane. The opening was sealed with cellophane tape, and the eggs were incubated at 38 °C until dissection. Embryos treated for 24 h with 2 μmol of carbachol or for 18 h with 5 μmol or 7.5 μmol had a survival rate of 25%. Only embryos alive at the time of dissection were used in these studies.

Isolation of cGIRK1 cDNA Clone—A 380-base pair fragment of GIRK1 was amplified from 20 μg of total chick brain RNA by reverse transcriptase with AMV-reverse transcriptase (Boehringer Mannheim) using a specific oligonucleotide (3’ primer), followed by polymerase chain reaction using Taq polymerase (Promega) with specific oligonucleotides (5’ and 3’ primers). The following oligonucleotides were synthesized by the University of Washington Molecular Pharmacology Facility: 5’ primer, 5’-TTCCAAAGTGGACTACTCCGAGTCCCA-3’; 3’ primer, 5’-CTCATGGGATCCGACATCATCTTGGTGGTC-3’A. An approximately 400-base pair fragment was amplified, isolated, and ligated into the BamHI and Sall sites of pGem3z (Promega). Sequence analysis using Sequenase (U. S. Biochemical Corp.) confirmed that we had cloned a 380-base pair fragment of chick GIRK1 (14).

Riboprobes—The GIRK1 RNA probe was generated from a 380-base pair polymerase chain reaction fragment described above corresponding to nucleotides 1027–1406 of the GIRK1 cDNA clone (14). SP6 and T7 RNA polymerases (Promega) were used to transcribe sense and antisense GIRK1 riboprobes, respectively. The chicken GIRK4 RNA probe was generated from a 445-base pair partial cDNA fragment (15) (Gen-Bank accession no. U71060), which displays 90% identity to rat GIRK4B. T3 and T7 RNA polymerases (Promega) were used to transcribe sense and antisense GIRK4 riboprobes, respectively. The probe for cm2 has been described previously (8). Labeled riboprobes were

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The abbreviations used are: mAChR, muscarinic acetylcholine receptor; G-protein, GTP-binding regulatory protein; GIRK, G-protein-coupled inwardly rectifying potassium channel; carbachol, carbamylcholine chloride; PBS, phosphate-buffered saline; TBST, Tris-buffered saline plus Tween 20.
separated from unincorporated nucleotides on a Sephadex G-50 RNA spin column (Boehringer Mannheim).

**Total Nucleic Acid Preparation—**Atria and ventricles were dissected, quick-frozen in liquid N2, and stored at −70 °C. Total nucleic acids were isolated from eight pooled atria and ventricles as described by McKnight et al. (16). Tissue was homogenized in 50 µg/ml proteinase K in 1 × SET (1% SDS, 5 mM EDTA, 10 mM Tris, pH 7.5) using a Polytron homogenizer and digested for 1 h at 45 °C. Samples were then phenol/chloroform/isoamyl alcohol-extracted, and nucleic acids were precipitated with 70% EtOH with 150 mM NaCl. Total nucleic acid pellets were chloroform/isoamyl alcohol-extracted, and nucleic acids were precipitated with 1 M phenanthroline, 1 M iodoacetamide, 400 mM phenylmethylsulfonyl fluoride, and 300 nM pepstatin A; all from Sigma) and prepared as described by Luetje et al. (18).

**Membrane Preparation for SDS-Gel Electrophoresis—**Pooled atria and ventricles (24–30 each) were glass homogenized in PBS with protease inhibitors (1 mM phenanthroline, 1 mM iodoacetamide, 400 µM phenylmethylsulfonyl fluoride, and 300 nM pepstatin A; all from Sigma) and resuspended in 0.1 M peptide corresponding to amino acids 482–498 of rat GIRK1. Renaissance Western blot chemiluminescent reagent (NEN Life Science Products) was used for detection of GIRK1 immunoreactivity. After Western blotting, the transfer membranes were stained with 0.01% Amido Black in 7% acetic acid for visualization of total protein. Relative levels of GIRK1 were determined by densitometry.

**Immunoblot Analysis of GIRK1 Expression—**Immunoblot analyses were carried out using an affinity-purified antibody raised against amino acids 482–498 of rat GIRK1. This antibody has been shown previously to be highly specific for GIRK1 as demonstrated by reactivity with cloned GIRK1, by reactivity with rat atrial but not ventricular cells, and by block of immunoreactivity by preincubation with the peptide antigen (19). 25 µg of the particulate fractions were subjected to SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to Immobilon-P transfer membrane (Millipore Corp.). Transfer membranes were blocked in 5% bovine serum albumin in TBST (Tris-buffered saline with 0.1% Tween 20) for 1 h at room temperature. After washing in TBST, transfer membranes were incubated first with 1 µg/ml affinity-purified anti-GIRK1 (19) and then with horseradish peroxidase-conjugated goat anti-rabbit IgG. Both primary and secondary antibodies were diluted in 1% bovine serum albumin in TBST and incubated at room temperature for 60 min. The specificity of the anti-GIRK1 antibody was tested by addition of 5 µg peptide corresponding to amino acids 482–498 of rat GIRK1. Renaissance Western blot chemiluminescent reagent (NEN Life Science Products) was used for detection of GIRK1 immunoreactivity. After Western blotting, the transfer membranes were stained with 0.01% Amido Black in 7% acetic acid for visualization of total protein. Relative levels of GIRK1 were determined by densitometry.

**RESULTS**

**GIRK mRNA Expression in Chick Atria and Ventricles—**We determined the amounts of GIRK1 and GIRK4 mRNA expression in embryonic day 9 chick atria and ventricles using solution hybridization. GIRK1 is expressed in atria at 1.8 ± 0.5 molecules/atrial cell (n = 6). Although mammalian ventricles do not express significant amounts of GIRK1 or GIRK4, GIRK1 was detected previously by RNase protection in chick ventricles (14). GIRK1 is expressed at lower levels in chick ventricles (0.5 ± 0.2 molecules/ventricular cell, n = 6). GIRK4 is expressed in atria at 2.6 ± 0.4 molecules/atrial cell (n = 6) with no expression detectable by solution hybridization in ventricles (< 0.2 molecules mRNA/cell).

**Time Course and Dose Dependence of Carbachol-induced GIRK mRNA Expression Decrease—**To investigate the effect of mACHr activation on GIRK1 and GIRK4 mRNA levels, we treated embryonic day 9 chick embryos with the agonist carbachol. Treatment of 9dE embryos with 2 µmol of carbachol in ovo resulted in a time-dependent decrease in the level of GIRK1 mRNA in atria (Fig. 1A). The level of GIRK1 mRNA was decreased to 48 ± 5% of control within 3 h of agonist treatment. Interestingly, no decrease in the level of GIRK1 mRNA in ventricles was observed after mACHr stimulation at all time points (Fig. 1B). GIRK4 expression also displayed a time-dependent decrease of specific mRNA with carbachol treatment (Fig. 1A). Levels of GIRK4 mRNA decreased to 69 ± 7% of control within the 2 h of carbachol treatment.

The dose dependence for the decrease in GIRK1 and GIRK4 mRNA was also determined. In embryos treated in ovo with 0.25–7.5 µmol of carbachol for 18 h, the expression of GIRK1 mRNA in atria decreased to 27 ± 7% of control at the maximum concentration tested, whereas GIRK4 mRNA expression decreased to 73 ± 6% of control levels at the highest concentration (Fig. 2A). Both GIRK1 and GIRK4 mRNA expression displayed a 30% decrease when treated with 0.5 µmol of carbachol. No further decrease in GIRK4 mRNA expression was detected with higher concentrations of carbachol. The expression of GIRK1 mRNA displayed a gradual decline with increasing concentrations of carbachol. The doses of agonist that cause decreases in GIRK mRNA are similar to those that cause carbachol-mediated down-regulation of mACHr number.
in ovo (7). The level of GIRK1 mRNA in ventricles was not decreased at any concentration of agonist (Fig. 2 B).

mAChR mRNA Down-regulation in Atria and Ventricles—As a control for the differential regulation of GIRK1 mRNA in atria and ventricles after mAChR activation, we determined the level of mAChR mRNA after in ovo carbachol treatment. Previous studies demonstrated that administration of carbachol to primary chick heart cell cultures results in decreased levels of mRNA encoding the m2 and m4 mAChRs (8). Solution hybridization was used to determine the amounts of m2 mAChR mRNA after treatment with 2 μmol of carbachol. With in ovo carbachol treatment, the level of mRNA encoding the m2 mAChR displayed time-dependent decreases in both atria and ventricles (Fig. 3) with a time course similar to those for GIRK1 and GIRK4 in atria.

Specificity of Carbachol-induced GIRK mRNA Decrease—The specificity of mAChR activation by carbachol was tested using the specific mAChR antagonist atropine. Administration of atropine is sufficient to block the carbachol-induced decrease in total mAChR number in chick heart (7) and the decrease in mAChR gene expression (8). Co-administration of 2 μmol of carbachol and 0.1 μmol of atropine to embryos in ovo blocked the decrease in mRNA for GIRK1 by 67% and GIRK4 by 100% (Fig. 1 A).

We also tested the reversibility of the decreases in GIRK mRNA by administering saturating doses of atropine (0.1 μmol) after GIRK mRNA levels were reduced by pretreatment with 2 μmol of carbachol for 3 h. An increase in the expression of both GIRK1 and GIRK4 mRNA was evident between 2 and 6 h after atropine treatment (Fig. 4). Further increases in both mRNAs proceeded gradually until control levels of expression were obtained after 24 h of atropine treatment. The block of GIRK mRNA decrease by atropine and the recovery of GIRK mRNA after subsequent administration of atropine demonstrate that the decrease in GIRK1 and GIRK4 mRNA levels is a result of activation of mAChR.

Carbachol-induced Decrease in GIRK1 Protein Expression—To test if the changes in K⁺ channel subunit mRNAs caused a decrease in GIRK protein levels, we investigated the effect of mAChR stimulation on the expression of the GIRK1 subunit. We determined relative levels of GIRK1 polypeptide using Western blots of crude particulate fractions from atria and ventricles probed with an antibody to rat GIRK1 (19). Consistent with previous reports (5), the antibody recognized a band of approximately 62 kDa in rat (Fig. 5A). The anti GIRK1 antibody recognized a polypeptide of 53 kDa in chick atria, which was present in much lower amounts in chick ventricle. As shown in Fig. 5B, immunoreactivity was greatly reduced by incubation of the antibody with excess peptide antigen. The smaller size of the GIRK1 immunoreactive band in chick compared with rat is consistent with the differences in the length of the coding regions from the two species. Atria from embryos treated with 2 μmol of carbachol for 24 h displayed a decreased amount of GIRK1 polypeptide. The 53-kDa immunoreactive band of GIRK1 in atria was decreased to 46 ± 12% (n = 3) of control in embryos treated with 2 μmol of carbachol. Thus, the decrease in GIRK1 mRNA levels is accompanied by a decrease in the level of GIRK1 polypeptide levels in carbachol-treated embryos.
DISCUSSION

The chick embryo is a convenient system for studying the regulation of the mACHR and effector molecules in vivo. At day 9, the embryonic chick heart beats spontaneously but has not developed functional parasympathetic or sympathetic innervation (20). The atria have functional mACHRs and respond to cholinergic agonists. Drugs administered to the embryo on the embryonic membrane are readily absorbed and do not disrupt the embryonic membranes or vascular system. This system has been used previously to demonstrate agonist induced decreases in mACHR number and function in vivo (7). Here, we have used this system to demonstrate that GIRK1 and GIRK4 are differentially expressed in atria and ventricles and that activation of mACHRs in the heart leads to decreased GIRK1 and GIRK4 mRNA in vivo.

Both solution hybridization and immunoblot analysis demonstrate that GIRK1 is expressed at higher levels in atria than in ventricles, consistent with previous Northern blot analysis (14). GIRK4, as in mammalian heart (5), is only detected in chick atria. This differential expression of GIRK1 and GIRK4 is consistent with cardiac physiology; muscarinic agonists cause activation of inwardly rectifying K⁺ channels in atria but not in ventricles. Native mACHR-gated inwardly rectifying K⁺ channels in heart are heteromultimers of GIRK1 and GIRK4 (5). Expression of cloned GIRK1 alone does not produce functional channels, and expression of GIRK4 alone results in currents unlike native atrial inwardly rectifying K⁺ channels (5). The lack of mACHR-activated K⁺ currents in chick ventricles is thus most likely a result of the inability of the low level of GIRK1 polypeptide to form a functional mACHR-gated K⁺ channel without its partner, GIRK4.

We have shown here that sustained activation of atrial mACHR in vivo can decrease the expression levels of GIRK1 mRNA and GIRK4 mRNA in a time and dose-dependent manner. These decreases are reversible when further mACHR stimulation is blocked by the muscarinic antagonist atropine. In addition, GIRK1 protein levels are also decreased in atria upon activation of mACHRs in vivo. This is the first direct demonstration of the down-regulation of a G-protein-coupled effector molecule by receptor activation in ovo. Although a preliminary report in abstract form (21) has reported a transient decrease in GIRK1 mRNA in cultured atrial cells after carbachol treatment, the results here demonstrate large and long lasting decreases in the levels of GIRK1 and GIRK4 mRNA as well as GIRK1 polypeptide levels after persistent mAChR activation in vivo. Interestingly, no decrease is detected in GIRK1 mRNA expression in ventricles after prolonged agonist treatment.

The differential regulation of GIRK1 mRNA expression in...
GIRK1 and GIRK4 are also expressed in the brain (27, 28) and may form heteromerultimers with other members of the GIRK family, GIRK2 and GIRK3 (29), to produce receptor-mediated changes in K⁺ conductance. Other G-protein-coupled receptors, such as opioid (30), cannabinoid (31), and serotonin (27) receptors, have been demonstrated to couple to the activation of GIRK1-containing potassium channels. This G-protein-coupled receptor-mediated down-regulation of GIRK1 and GIRK4 could represent an important pathway for regulating physiological responses in the heart and the nervous system.

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