Subtype-specific Regulation of Muscarinic Receptor Expression and Function by Heterologous Receptor Activation*

Darrell A. Jackson and Neil M. Nathanson†

From the Department of Pharmacology, University of Washington, Seattle, Washington 98195-7750

Incubation of cultured embryonic chicken heart cells with the \(\beta\)-adrenergic agonist isoproterenol resulted in a dose-dependent increase in the number of mACHR on the surface of intact cells. The isoproterenol-mediated increase in mACHR number was time dependent and reached a maximum by 48 h. Chick heart cells treated with isoproterenol exhibited a greater than 6-fold increase in the sensitivity for carboclophil-mediated inhibition of adenyl cyclase activity as compared to control. Stimulation of cultured heart cells for 24 h with isoproterenol resulted in a 25–35% increase in cm2 mRNA levels as compared to control cm2 mRNA levels. In contrast, the level of cm4 mRNA was not significantly affected by isoproterenol treatment. cm2 mRNA levels were maximally elevated by 15 h following isoproterenol stimulation and remained elevated for up to 72 h. Incubation of cells with isoproterenol in the presence of Rp-cAMP, an inhibitor of cAMP-dependent protein kinase, blocked the increase in the level of cm2 mRNA. Thus, prolonged activation of \(\beta\)-adrenergic receptor number, but also in a significant reduction in the number of mRNA encoding the cm2 and cm4 mACHRs (Habecker and Nathanson, 1992; Habecker et al., 1993). In addition to the agonist-induced down-regulation of mACHRs in the chick heart, activation of adenase (Ad1) and angiotensin II (Ang1) receptors has also been shown to affect chick heart mACHR number and mRNA levels (Habecker and Nathanson, 1992). Chick cardiac cells also express \(\beta\)-adrenergic receptors which mediate stimulation of adenyl cyclase activity. Stimulation of mACHRs results in inhibition of \(\beta\)-adrenergic stimulation of cardiac contractility due at least in part through inhibition of adenyl cyclase (Hartzel, 1988). We report here that persistent stimulation with the \(\beta\)-adrenergic receptor agonist isoproterenol increases mACHR number and muscarinic responsiveness in chick heart cells due to a selective increase in cm2 mRNA levels with no effect on cm4 mRNA levels.

Muscarnic acetylcholine receptors (mACHRs) are members of a large family of transmembrane spanning receptors that couple to guanine nucleotide-binding proteins (G-proteins) upon agonist activation. Five different genes encoding the mammalian mACHRs subtypes (m1-m5) have been identified (Kubo et al., 1986a, 1986b; Peralta et al., 1987a, 1977b; Bonner et al., 1987, 1988; Braun et al., 1987; Shapiro et al., 1988). The chick heart expresses at least three mACHR subtypes (cm2, cm3, and cm4) that share a high degree of homology to the mammalian m2, m3, and m4 mACHR (Tietje et al., 1990; Tietje and Nathanson, 1991; Gadbut and Galper, 1994). Agonist activation of mACHRs has been shown to result in a variety of physiological and biochemical events that include inhibition of adenyl cyclase (Nathanson et al., 1978), stimulation of guanylyl cyclase (Renaud et al., 1980), stimulation of phospholipase C (Brown and Masters, 1984), and alteration of ion channel conductances (Galper et al., 1982; Hunter and Nathanson, 1986). Activation of phospholipase C results in the generation of the second messengers diacylglycerol and inositol triphosphate (Orellana and Brown, 1985) and subsequent activation of protein kinase C and increase in intracellular calcium levels (Hirasawa and Nishisuka, 1985). In the continued presence of acetylcholine or other agonists, mACHR in the heart and other tissues undergo sequestration and a subsequent decrease in receptor number (Nathanson, 1987). Long term treatment of embryonic chick heart cells with muscarinic agonists results not only in a down-regulation of muscarinic receptor number, but also in a significant reduction in the levels of mRNA encoding the cm2 and cm4 mACHRs (Habecker and Nathanson, 1992; Habecker et al., 1993). In addition to the agonist-induced down-regulation of mACHRs in the chick heart, activation of adenase (Ad1) and angiotensin II (Ang1) receptors has also been shown to affect chick heart mACHR number and mRNA levels (Habecker and Nathanson, 1992). Chick cardiac cells also express \(\beta\)-adrenergic receptors which mediate stimulation of adenyl cyclase activity. Stimulation of mACHRs results in inhibition of \(\beta\)-adrenergic stimulation of cardiac contractility due at least in part through inhibition of adenyl cyclase (Hartzel, 1988).

We report here that persistent stimulation with the \(\beta\)-adrenergic receptor agonist isoproterenol increases mACHR number and muscarinic responsiveness in chick heart cells due to a selective increase in cm2 mRNA levels with no effect on cm4 mRNA levels.

**Experimenal Procedures**

Materials—White leghorn chicken eggs were obtained from H & N International (Redmond, WA) and maintained in a humidified 38°C incubator until the ninth day of incubation. \(\text{[H]}^{3} \text{H} \text{QNB} (35-44 \text{Ci/mmol}) \) and \(\text{[H]}^{3} \text{H} \text{NMS} (85 \text{Ci/mmol}) \) were obtained from Amersham Corp. \(\text{[H]}^{3} \text{H} \text{xAMP (36 Ci/mmol)} \) was from ICN, and \(\alpha-\text{[32P]} \text{UTP (800 Ci/mmol)} \) was obtained from DuPont NEN and \(\alpha-\text{[3H]} \text{UTP (41 Ci/mmole)} \) was from Amersham. The ion-exchange resin AG50W-X4 (200– 400 mesh, hydrogen form) used in the cAMP accumulation assay was from Bio-Rad. Cell culture media were purchased from Life Technologies, Inc. and Sigma. All other materials were purchased as described previously (Halvorsen and Nathanson, 1981).

Cell Culture—Heart cells from 9-day embryonic chicken were prepared as described by Subers and Nathanson (1988) in serum-free defined medium containing 98% M-199, 1% penicillin-streptomycin (100 units/ml and 100 \(\mu\)g/ml final concentrations, respectively), insulin (5 \(\mu\)g/ml), transferrin (5 \(\mu\)g/ml), sodium selenite (5 ng/ml), testosterone (10 \(\mathrm{nm}) \) final concentration), and triiodothyronine (3 \(\mathrm{nm}) \) final concentration). Cardiac cells were plated at a density of 1.0 \(\times\) 10^5 cells/cm² on 100-mm plastic tissue culture dishes and maintained in a 5% CO₂ environment at 37°C. Isoproterenol, dissolved in 10 \(\mu\)M ascorbic acid, was added once daily, beginning 24 h after culture preparation and until 72 h or otherwise noted. The media was changed on the third day, and assays were performed on the fourth day of culture.

Binding Assay in Membrane Homogenates—The binding of \(\text{[H]}^{3} \text{H} \text{QNB} to mACHR in crude membrane homogenates was performed according to the method of Halvorsen and Nathanson (1981). Binding of \(\text{[H]}^{3} \text{H} \text{NMS} to cell surface mACHR on intact chick heart cells in culture was performed as described by Nathanson (1983). In all experiments, nonspe-
Control (10 μM) presented as the mean ± S.D. from three separate experiments which each had from six to nine independent determinations. The increase in [3H]NMS binding is expressed as the percent increase compared to control (10 μM ascorbic acid). The mean value of [3H]NMS binding to control heart cells was 960 ± 40 fmol/100-mm plate.

Specific binding was determined as amount of [3H]QNB binding remaining in the presence of 1 μM atropine.

cAMP Accumulation Assays—Chick heart cell cultures were prepared as described above on 60-mm tissue culture plates. Carbachol-mediated inhibition of forskolin-stimulated cAMP accumulation was measured as described by Subers and Nathanson (1988).

RNA Probe Construction—Antisense and sense RNA probes were to a BamH1/Xba1 fragment encoding the subtype-specific third cytoplasmic loop of the cm2 receptor (Tietje et al., 1991) and a Balt/Smal fragment encoding the third cytoplasmic loop of the cm4 receptor (Tietje and Nathanson, 1991). Antisense probes were synthesized with [α-33P]UTP and sense RNA for standards with [α-33P]UTP.

Isolation of RNA from Chick Heart Cells—Total cellular RNA was isolated as described by Peppel and Baglioni (1990) and stored in 2-propanol at -20 °C until use. Samples were resuspended in 0.1% SDS, and the RNA concentration was determined by UV spectrophotometer.

Solution Hybridization—Quantitation of cm2 and cm4 mRNA was measured by hybridization of RNA samples with antisense probes as described by Habecker and Nathanson (1992).

RESULTS

β-adrenergic-mediated Increase in mAChR Numbers—Chronic treatment of embryonic heart cells in culture with a high concentration of β-adrenergic agonist has been reported to result in an increase in mAChR number in membrane homogenates (Reithmann et al., 1992). However, because measurements of mAChRs were performed using the membrane-permeable muscarinic antagonist [3H]QNB, it was not demonstrated whether chronic β-adrenergic stimulation resulted in an increase in the number of mAChRs on the cell surface. To determine whether β-adrenergic stimulation resulted in an increase in mAChRs numbers on the surface of intact cells, we used the membrane-impermeable antagonist [3H]NMS. Treatment of cultured embryonic chick hearts in defined media with isoproterenol for 72 h resulted in a dose-dependent increase in the number of mAChR on the surface of intact cells as measured with the membrane-impermeable muscarinic antagonist [3H]NMS (Fig. 1). We also observed similar increases in the number of total cellular mAChRs as measured by the binding of the lipophilic muscarinic antagonist [3H]QNB to membrane homogenates (Fig. 2).

To ensure that the increase in mAChR numbers by isoproterenol stimulation was due to activation of β-adrenergic receptors, we pretreated the cells with the β-adrenergic antagonist nadolol. Pretreatment of embryonic chick cell cultures with nadolol prior to the addition of 100 nM isoproterenol significantly attenuated the increase in mAChR number (Fig. 2A). Treatment of cells with nadolol alone did not produce a significant effect on mAChR number as compared to control (Fig. 2A). In addition, we also observed a time-dependent increase in mAChR numbers in cells stimulated by isoproterenol. Muscarinic receptor number began to increase by 24 h and was maximally elevated by 48 h after isoproterenol stimulation (Fig. 2B).

CAMP Accumulation Assay—In order to determine if the increase in mAChR number following β-adrenergic agonist stimulation resulted in an increased functional responsiveness to muscarinic agonists, we examined the ability of the mAChR
to inhibit adenylyl cyclase activity. The concentration-response curves for carbachol-mediated inhibition of forskolin stimulation of cyclic AMP formation demonstrated that chick heart cells treated with 10 μM isoproterenol exhibited an increased physiological sensitivity to muscarinic agonists (Fig. 3). Forskolin-mediated increase in cAMP formation was maximally inhibited 70% by carbachol in both isoproterenol-stimulated and vehicle-treated heart cells (Fig. 3). However, isoproterenol-treated cells exhibited greater than a 6-fold increase in the sensitivity for carbachol-mediated inhibition of adenylyl cyclase as compared to control (EC50 values of 3.0 × 10^{-8} and 1.9 × 10^{-7} M, for isoproterenol-treated and control cells, respectively).

Solution Hybridization Analysis of RNA Isolated from Iso-proterenol-treated Cells—We examined if the increase in mAChR number was due to an increased level of mRNA encoding the mAChR. Cultured chick heart cells express two main mAChR subtypes, cm2 and cm4. The levels of cm2 and cm4 mRNA were quantitated by solution hybridization using subtype-specific riboprobes as described by Habecker and Nathanson (1992). Interestingly, isoproterenol stimulation of chick heart cell cultures resulted in a subtype-selective increase in the level of mAChR mRNA. Incubation with 100 nM isoproterenol for 24 h resulted in a 27% increase in cm2 mRNA levels as compared to control cm2 mRNA levels (1.1 ± 0.1 × 10^{6} to 1.4 ± 0.1 × 10^{6} molecules/μg RNA, p = 0.02). In contrast, the level of cm4 mRNA was not significantly affected by isoproterenol treatment (6.5 ± 0.1 × 10^{6} and 6.8 ± 0.1 × 10^{6} molecules/μg RNA, p > 0.1). cm2 mRNA levels were maximally elevated by 15 h following isoproterenol stimulation (Fig. 4A) and remained elevated for up to 72 h.

We used the competitive inhibitor of cAMP-dependent protein kinase, Rp-cAMP (Botelho et al., 1988), to test whether the increase in cm2 mRNA levels by isoproterenol was a result of activation of cAMP-dependent protein kinase. Incubation of cells with isoproterenol in the presence of Rp-cAMP did not lead to an increase in the level of cm2 mRNA (Fig. 4B), demonstrating that the β-adrenergic receptor-mediated increase in mAChR expression is mediated through cAMP-dependent protein kinase.

**DISCUSSION**

This work demonstrates that chronic stimulation of cultured chick heart cells β-adrenergic receptors results in an increased mAChR number and muscarinic responsiveness in chick heart cells due to a selective increase in cm2 mRNA levels with no effect on cm4 mRNA levels. Chick β-adrenergic receptors appear to be pharmacologically similar to the mammalian β-1 subtype (Port et al., 1992). Isoproterenol-mediated activation of these β-adrenergic receptors results in stimulation of adenylyl cyclase activity and a subsequent increase in cAMP accumulation (Port et al., 1992). Pretreatment of cultured heart cells with the cAMP-dependent protein kinase inhibitor, Rp-cAMP, blocked the isoproterenol-mediated increase in cm2 mRNA levels. Thus, the isoproterenol-mediated increase in cm2 mRNA levels involves activation of cAMP-dependent protein kinase. Interestingly, the isoproterenol-mediated increase in mAChR numbers was due to a differential effect on cm2 and cm4 mRNA levels. Although cm2 mRNA levels were maximally increased by 25–35%, cm4 mRNA did not exhibit any changes following isoproterenol stimulation. Prolonged agonist activation of chick heart mAChRs results in a subsequent decrease in both...
mACHR number (Galper and Smith, 1980) in mRNA levels encoding both the cm2 and cm4 receptors (Habecker and Nathanson, 1992). Treatment of chick heart cells with muscarinic agonists causes both inhibition of adenyl cyclase and stimulation of phospholipase C (Brown and Brown, 1984). Coupling of the mACHR to both second messenger pathways is required for agonist-mediated regulation of cm2 and cm4 mRNA. Simultaneous activation of the Ad1 receptor, which results in inhibition of adenyl cyclase in the chick heart, and AngII receptors, which activates phospholipase C in the chick heart, also resulted in a reduction in mACHR numbers and cm2 and cm4 mRNA levels (Habecker and Nathanson, 1992). These results indicate that there is a complex role of second messenger pathways in the regulation of mACHR gene expression. While the mRNA encoding the cm2 and cm4 receptors are regulated in a similar fashion by activation of mACHR or Ad1 receptors and AngII receptors, the expression of cm2 and cm4 mRNAs are differentially regulated by β-adrenergic receptor activation.

Reithmann et al. (1992) previously reported that chick cardiac cells which were treated with β-adrenergic agonists did not exhibit an increased magnitude of inhibition of adenyl cyclase activity in response to a single high concentration of carbachol. However, because of the presence of spare receptors, an increase in mACHR number should lead not to an increase in extent of inhibition but to a decrease in the concentration of carbachol required for inhibition of adenyl cyclase activity (Halvorsen and Nathanson, 1981; Hunter and Nathanson, 1986). Indeed, we show here that the isoproterenol-mediated increase in mACHR numbers leads to a greater responsiveness to carbachol-mediated inhibition of forskolin stimulation of adenyl cyclase (Fig. 3).

We have shown here that chronic stimulation of β-adrenergic receptors by isoproterenol causes an increase in mACHR numbers in heart cells. In addition, these cells exhibit an increased sensitivity for mACHR-mediated inhibition of forskolin stimulation of adenyl cyclase. Furthermore, we have shown that β-adrenergic activation results in a selective increase in mRNA levels encoding the cm2 mACHR subtype. This increase in cm2 mRNA levels is dependent upon activation of cAMP-dependent protein kinase.

Long term treatment with β-adrenergic antagonists has been shown to induce an increase in β-adrenergic receptor number and a concomitant decrease in muscarinic m2 receptor number and functional responsiveness in mammalian heart (Motomura et al., 1990; Marquetant et al., 1992). These changes in neurotransmitter receptor levels are likely to contribute to the adverse clinical effects of abrupt withdrawal of chronic β-adrenergic antagonist administration (Prichard et al., 1983; Fishman, 1987). The regulation of mACHR expression by β-adrenergic receptors thus is not only an interesting example of the regulation of neurotransmitter receptors by heterologous receptor activation but also has important clinical implications.

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