A Novel M₃ Muscarinic Acetylcholine Receptor Is Expressed in Chick Atrium and Ventricle*

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Prior studies have suggested that heart expresses only the M₁ isoform of the muscarinic receptor (Peralta et al., 1987). From the Cardiovascular Division, Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts 02115. The abbreviations used are: MAchR, muscarinic acetylcholine receptor; CHO, Chinese hamster ovary; QNB, quinuclidinyl benzilate; PLC, phospholipase C; IP, inositol phosphate; IP₃, inositol trisphosphate; bp, base pair(s); PCR, polymerase chain reaction; HHS, hexahydrodilafodifenidol.

The muscarinic acetylcholine receptor (MAchR) belongs to the receptor superfamily which includes adrenergic (Dixon et al., 1986), dopaminergic (Bunzow et al., 1988), and serotonin receptors (Julius et al., 1988). All of these receptors are characterized by the presence of seven transmembrane-spanning domains, and the interaction of the third intracellular loop of these receptors with a heterotrimeric guanine nucleotide-binding protein (G-protein). Muscarinic receptors regulate the rate and force of contraction of the heart by mediating the inhibition of adenyl cyclase (Tang and Gilman, 1992), stimulating inositol phosphate and diacylglycerol production (Barnett et al., 1990; Masters et al., 1985), and activating acetylcholine-sensitive K⁺ channels (Logothetis et al., 1987) and the production of cyclic GMP. The physiologic response to the binding of hormones to these receptors is transduced to effectors via the interaction of the receptor with a G-protein.

Muscarinic cholinergic receptors have been shown by molecular cloning to be coded for by intronless genes. Five different isoforms of muscarinic receptors have thus far been cloned and designated M₁, M₂, M₃, M₄, and M₅ (Bonneur et al., 1987, 1988). These receptor subtypes can be distinguished pharmacologically by characteristic competition binding patterns between a radiolabeled antagonist and specific muscarinic ligands (Buckley et al., 1989).

Expression of clones coding for isoforms of the muscarinic receptor has demonstrated that the M₁ and M₃ isoforms are characterized biochemically by stimulation of a large inositol phosphate response while having a small stimulatory effect on adenylate cyclase activity. The M₂ and M₃ isoforms of the receptor are linked to an inhibition of adenylyl cyclase activity and only a modest stimulation of inositol phosphate release (Peralta et al., 1988). M₂ receptors appear to be coupled in a manner similar to M₁ and M₃. MAchR subtypes are further characterized by their tissue specific expression. In cardiac tissue only the M₃ isoform of the muscarinic receptor had been demonstrated (Bonneur et al., 1987; Peralta et al., 1987). However, recently the chick atrium has been shown to be unique in that it expresses mRNAs coding for both the M₃ and M₅ isoforms of the muscarinic receptor (Tietje and Nathanson, 1991).

The M₅ receptor transcribed into Xenopus oocytes has been shown to be coupled to inositol phosphate production via a pertussis toxin-sensitive G-protein, whereas the M₃ receptor has been shown to be coupled to inositol phosphate production via a pertussis toxin-insensitive G-protein. We have demonstrated previously that muscarinic-stimulated inositol phosphate release in the chick atrium appears to be coupled to both a pertussis toxin-sensitive and pertussis toxin-insensitive G-
protein (Barnett et al., 1990). Hence an M3 receptor in the chick atrium might be coupled to a pertussis toxin-insensitive fraction of IP3 production, and the M4 or M5 receptor may be responsible for the pertussis toxin-sensitive fraction of IP3 production in the chick atrium. To determine whether M3 receptors are expressed in the atrium, we cloned the M3 muscarinic receptor from chick and determined its biochemical and pharmacological characteristics in stably transfected CHO cells. We found that the chick M3 receptor was coupled to a pertussis toxin-insensitive stimulation of inositol phosphate release. Using RNase protection analysis we further demonstrated that mRNA coding for this chick M3 receptor was expressed in the chick heart primarily in the atrium.

**MATERIALS AND METHODS**

**Isolation and Sequencing of Clones—**A cDNA library from chick brain 17 days in ovo, constructed in the a-ZAP vector (Stratagene, gift of K. Takeyasu), was screened at low stringency with a full-length human M3 muscarinic acetylcholine receptor cDNA (gift of E. Peralta). The human M3 cDNA probe was labeled by the random primer method with [32P]dCTP (Feinberg and Vogelstein, 1984). The hybridization and washing conditions were as described (Sambrook et al., 1977). nylon membranes were hybridized to the probe in 10% dextran sulfate, 40% formamide, 1 x SSC, 7 ma Tris, pH 7.6, 0.8% 100 ug/ml of salmon sperm DNA (2 mg/ml) for 24 h at 37°C. The membranes were washed three times in 2 x SSC, 0.1% SDS for 5 min at 35°C followed by three washes in 0.2 x SSC, 0.1% SDS for 15 min at 48°C. From the primary screen nine cDNA clones were isolated. Two clones coded for the same unique full-length receptor protein. This clone was completely sequenced on both strands using a combination of subcloned restriction fragments and synthetic oligonucleotide primers, by the dideoxy chain termination method (Sanger et al., 1977). Other clones isolated included cDNAs coding for full-length chick M3 and M4 receptors which were 100% identical to those cloned by Tietje et al. (1990) and Tietje and Nathanson (1991).

**RNase Protection Assays—**Total RNA was isolated from 17-day in ovo chick tissues using guanidium CsCl centrifugation (Sambrook et al., 1989). For the generation of M3 RNase protection probes, an XhoI fragment of the M3 cDNA (nucleotide -97 to 1193) was subcloned into Bluescript II and the construct digested with BarnHI (see restriction map (Tietje et al., 1991)). Using T7 RNA polymerase (Boehringer Mannheim) in the presence of [32P]UTP, this linearized template gave a 449-bp antisense riboprobe to a portion of mRNA coding for the second extracellular and third intracellular loops (see restriction map, Tietje and Nathanson, 1991).

For the M3 RNase protection probe, a 350-bp template was generated by PCR amplification spanning bases -4 to 346 (see restriction map (Tietje et al., 1990)). The PCR product was subcloned into pBluescript II SK+, sequenced, and then linearized with XbaI. Using T3 RNA polymerase (Boehringer Mannheim), in the presence of [32P]UTP, this template gave a 350-bp antisense riboprobe to a portion of mRNA coding for the amino terminus of M3.

For the M3 RNase protection probe a 636-bp template was generated by PCR amplification spanning bases 1080-1716 (see Fig. 1). The PCR product was subcloned into pBluescript II SK+, sequenced, and linearized with BglII. Using T7 RNA polymerase (Boehringer Mannheim), in the presence of [32P]UTP, this template gave a 636-bp antisense riboprobe to a portion of mRNA coding for the second intracellular loop and the third extracellular loop of the M3 receptor. RNase protection analysis was performed as described (Ausubel et al., 1987a). Following hybridization of the probes to total RNA, the samples were treated with ribonuclease and analyzed by polyacrylamide gel electrophoresis on 6% gels containing areas followed by autoradiography.

**Stable Expression of the Chick M3 Muscarinic Receptor in CHO Cells—**The Chinese hamster ovary cell line CHO-GRA, which is dihydrofolate reductase-deficient (a gift of Randall Kaufman), was grown in minimal essential medium supplemented with 10% fetal calf serum, 10 μg/ml adenosine, 10 μg/ml deoxyadenosine, and 10 μM thymidine in an environment of 5% CO2 at 37°C. To optimize translation of the M3 MACHR cDNA, 341 of the 358 bp 5' of the first in-frame ATG were deleted before ligation into a dihydrofolate reductase containing expression plasmid pKD (gift of Randall Kaufman). To generate this construct a combination of subcloned restriction fragments was deleted from the 5' end of the M3 cDNA by cleavage at the NcoI site at bp 188 (see Fig. 1). This fragment was replaced by ligating a 205-bp PCR product spanning bp 17 to 188,
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**FIG. 1.** Restriction map and nucleotide sequence of the chick M₃ muscarinic receptor cDNA. A, restriction map of the 2.4-kilobase pair cDNA coding for the chick M₃ muscarinic receptor. Darkened bars define the coding region. B, the nucleotide sequence and deduced amino acid sequence of the chick M₃ muscarinic receptor is shown. The first in frame ATG is depicted. The protein shares an 87 and 86% amino acid identity with the human and rat M₃ muscarinic receptor respectively. The protein contains 39 amino acids on the amino terminus not present in human or rat M₃ receptors. Furthermore, the chick receptor contains additional amino acids beginning at amino acid 89 which are not found in either the human or rat M₃ receptor. The overall high degree of identity to M₃ receptors in other species suggested that this cDNA coded for the chick form of the M₃ muscarinic receptor.

**Legend Binding in CHO Cells Transfected with the M₃ Muscarinic Receptor—CHO cells were transfected by electroporation with the M₃-pED muscarinic receptor construct as described under “Materials and Methods.” After 12 days in selective media, 10 colonies were picked and grown on individual plates. The levels of muscarinic receptor expressed in these clonal lines ranged from 100 fmol/mg to 4 pmol/mg of protein as determined by binding of the muscarinic antagonist [³H]QNB. The pharmacological and biochemical characterisation of the M₃ receptor was carried out in the clonal cell line CHO-CM3 which expressed the M₃ muscarinic receptor at approximately 100 fmol/mg of protein. [³H]QNB binding to membranes of these cells was saturable and demonstrated a high degree of specificity (Fig. 3a) with 89% specific binding at 0.3
nm [3H]QNB. Scatchard analysis of saturation binding data gave a straight line consistent with binding to a single class of receptor sites with a $K_d$ of 110 pm and a $B_{max}$ of 140 fmoI/mg of protein. No significant binding of [3H]QNB was observed in nontransfected cells at any concentration of [3H]QNB studied. The mean of three experiments similar to that shown in Fig. 3b gave a $K_d$ value of 76 ± 17 (±S.E., n = 3) pm and $B_{max}$ of 116 ± 14 (± S.E., n = 3) fmoI/mg of protein. This $K_d$ for [3H]QNB is consistent with observations reported previously for chicken heart (Galper et al., 1977; Halvorsen and Nathanson, 1981).

Muscarinic receptor subtypes demonstrate characteristic affinities for the binding of different muscarinic antagonists. Hence we characterized the binding properties of the $M_2$-like muscarinic receptor in CHO-CM3 cells using competition binding studies between a fixed concentration of [3H]QNB and various levels of hexahydrosalifendil, pirenzepine, and methoctramine. The data summarized in the typical curves presented in Fig. 4 demonstrate a rank order of potency of HHSD > pirenzepine > methoctramine for binding to the CHO-CM3 membranes. Analysis of these competition binding data by the method of Munson and Rodbard (1980) demonstrated that the data from each curve gave the best fit to a single-site model. The mean of data from three experiments similar to that in Fig. 4 demonstrated that HHSD, pirenzepine, and methoctramine bind to the receptor with $K_d$ values of 16 ± 3 (±S.E., n = 3) nM, 383 ± 47 (±S.E., n = 3) nM, and 533 ± 185 (±S.E., n = 3) nM, respectively. Comparison with the relative affinities for binding of these antagonists to mammalian muscarinic receptors expressed in CHO cells (Buckley et al., 1989) is most consistent with binding to an $M_3$ subtype of the muscarinic receptor.

Characterization of the Physiologic Response to Muscarinic Stimulation in CHO-CM3 Cells—The effect of carbachol on levels of cAMP production in CHO-CM3 cells was studied. In a typical experiment summarized in Fig. 5, incubation of CHO-CM3 cells with concentrations of carbachol from 10^{-4} to 10^{-3} M resulted in a 16-fold increase in cyclic AMP accumulation from 100 to 156 pmol/mg of protein. Data from two experiments similar to that shown in Fig. 5 demonstrate a mean increase in cAMP levels from a control level of 98 ± 2.0 (±S.E., n = 2) pmol/mg of protein to 156 ± 2.0 (±S.E., n = 2) pmol/mg of protein at 10^{-3} carbachol. Furthermore, incubation with 100 µM forskolin for 5 min resulted in a 5.7-fold stimulation of cyclic AMP accumulation, and concentrations of carbachol between 10^{-4} and 10^{-3} M increased levels of forskolin-stimulated cAMP production by 2-fold from 440 ± 10 (±S.E., n = 2) pmol/mg of protein to 826 ± 137 (± S.E., n = 2) pmol/mg of protein.

Production of Inositol Phosphate in Response to Muscarinic Stimulation—In order to determine whether the mechanism of coupling of the $M_3$ receptor to inositol phosphate in CHO-CM3 cells was characteristic of that reported for $M_3$ receptors cloned...
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Fig. 6. Inositol phosphate production in CHO-CM3 cells is pertussis toxin-insensitive. CHO-CM3 cells were grown to 80% confluence and incubated for 24 h with 1.25 μCi/ml [³²P]NAD and ADP-ribosylation determined as described under "Materials and Methods." The data are plotted as a percent of stimulation in control cells cultured in the absence of pertussis toxin. a, effect of pertussis toxin on carbamylcholine-stimulated inositol phosphate release. Cells prepared as described above were incubated for 16 h with the indicated concentrations of pertussis toxin. Cells were washed and incubated for 25 min with 10⁻³ M carbamylcholine. Data are plotted as a percent of stimulation in control cells cultured in the absence of pertussis toxin. The mean of data from four experiments similar to that in Fig. 6a demonstrated a 3.2-fold increase in inositol phosphate level from a basal level of 5918 ± 564 (±S.E., n = 4) cpm to 18,815 ± 1661 (±S.E.) cpm, with an EC₅₀ of 7.0 ± 2.9 (±S.E.) μM. These results are consistent with those reported for the human M₃ muscarinic receptor stably transfected into mammalian cells in which muscarinic stimulation produced a modest increase in cyclic AMP levels and a larger increase in inositol phosphate production (Peralta et al., 1988).

To determine whether muscarinic stimulation of inositol phosphate production in CHO-CM3 cells was coupled to the M₃ muscarinic receptor via a pertussis toxin-sensitive G-protein, CHO-CM3 cells were incubated for 16 h in the presence of various concentrations of pertussis toxin, and the effects of 10⁻³ M carbamylcholine on levels of inositol phosphate release were determined. Incubation of cells with pertussis toxin had no effect on the ability of carbamylcholine to stimulate inositol phosphate production. Data summarized in Fig. 6b demonstrated that at all concentrations of pertussis toxin studied, muscarinic stimulation of inositol phosphate production was the same as muscarinic stimulation of inositol phosphate production in control cells cultured in the absence of pertussis toxin. Thus carbamylcholine (10⁻³ M) stimulated inositol phosphate production 3.1-fold above basal following incubation with concentrations of pertussis toxin as high as 10 ng/ml.

One possible explanation for this lack of sensitivity of carbamylcholine-stimulated inositol phosphate production to pertussis toxin is that at the concentrations of pertussis toxin studied, ADP-ribosylation of CHO-CM3 cell G-proteins by endogenous NAD was incomplete. In order to rule out this possibility CHO-CM3 cells were incubated for 15 h with 10.0 ng/ml of pertussis toxin and the ability of pertussis toxin to catalyze the incorporation of [³²P]NAD into a 41-kDa protein in homogenates of these cells was determined by polyacrylamide gel electrophoresis and autoradiography as described under "Materials and Methods." Under these conditions cells demonstrated a complete loss of incorporation of [³²P]NAD into a 41-kDa band (Fig. 6c). The addition of Lubrol (0.1%) to homogenates of cells which were pretreated with pertussis toxin had no effect on the level of incorporation of [³²P]NAD, suggesting that the absence of pertussis toxin substrates in these homogenates was not due to sequestration.

**Tissue Distribution of Muscarinic Receptor mRNA by RNase Protection Analysis.—**Because of the low levels of expression of muscarinic receptor mRNAs, it was necessary to use RNase protection to determine the relative levels of receptor mRNAs in chick tissues. Total RNA was isolated from chick brain, atria, ventricle, and skeletal muscle of chick embryos 17 days in ovo and hybridized to antisense riboprobes generated from the chick muscarinic receptor constructs described under "Materials and Methods." The M₂, M₃, and M₄ riboprobes protected fragments of the predicted sizes (see "Materials and Methods"). Although the M₃ receptor was expressed at the highest levels in the brain, data summarized in Fig. 7 demonstrate the unique finding that the M₃ receptor is also expressed in the heart with significantly higher levels in the atrium than in the ventricle. M₄ was not expressed in skeletal muscle. The M₃ receptor demy of pertussis toxin. Lane 3, control cells in which homogenates were incubated with [³²P]NAD in the absence of pertussis toxin.
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These data demonstrate that we have cloned a muscarinic receptor from chick with a high degree of identity to M₃ receptors cloned from human and rat. This receptor when expressed in CHO cells binds muscarinic antagonists in a pattern characteristic of M₃ receptors. The receptor is coupled to an increase in cAMP production and to a pertussis toxin-insensitive increase in inositol phosphate production which is also characteristic of M₃ receptors. Studies further demonstrate the unique finding that unlike rat heart (Peralta et al., 1987; Bonner et al., 1988) chick atrium and ventricle both express the M₃ form of the muscarinic receptor.

The M₃ muscarinic receptor from chick is a 639-amino acid protein with 87% homology to rat and human M₃ muscarinic receptor subtypes. The major differences in amino acid sequence occur within the predicted third intracellular loop and

DISCUSSION

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was subjected to RNase protection analysis as described under "Materials and Methods." The full-length riboprobes undigested with ribonuclease are shown in the left-hand lanes. These probes contained an additional 55, 72, and 47 nucleotides of Bluescript II on the 5' end of each transcript of M₃, M₄, and M₅, respectively, which were not protected from ribonuclease. DNA sequencing reactions run as standards in each experiment (not shown) were used to determine fragment sizes which are labeled on the left of each panel. Antisense riboprobes were hybridized to 20 μg of total RNA for M₄ and M₅ and 10 μg of total RNA for M₃. Autoradiograms represent exposures of 52, 42, and 29 h for M₃, M₄, and M₅ respectively. b, atrium and ventricle from hearts of embryos 14 days in ovo were cultured as described under "Materials and Methods." On the 3rd cul decid RNAs, cells were harvested and total RNA prepared as described for intact tissue. Total RNA was subjected to RNase protection analyses as described above. M₃, M₄, and M₅ antisense riboprobes were hybridized to 10, 20, and 20 μg of atrial and ventricular RNAs, respectively. The autoradiograph for M₃ was exposed for 24 h and M₄ and M₅ for 72 h.
in the amino acid terminus. The third intercellular loop of chick M₃ is 77% identical to that in the human M₃ receptor. Both the M₃ and M₄ muscarinic receptors in the chick have longer amino termini than either of their rat or human homologs. The chick M₄ muscarinic receptor is 13 amino acid longer than either the human or rat M₄ (Tietje et al., 1990), and the chick M₃ receptor is 39 amino acid longer than either the human or rat M₃ receptor (Fig. 2). What effect these extended sequences play in the functioning of the protein in the chick is unknown.

The chick M₃ receptor expressed in CHO cells demonstrated the rank order of potency for competition of muscarinic ligands with the binding of [³H]QNB of HHSD to pertussin-sensitive muscarinic receptor subtypes from other species. The small differences between the binding affinities of the chick M₃ receptor expressed in CHO cells and the values published for the human receptors may reflect the differences in amino acids between the chick and human receptors noted in Fig. 2.

Lechleiter et al. (1990) have demonstrated that the specificity of a muscarinic receptor for coupling to PLC activity via a given G-protein is determined at least in part by the amino-terminal 18 amino acids of the third intracellular loop. These amino acids are identical between chick, human, and rat M₃ receptors (Fig. 2). This is consistent with the finding that muscarinic stimulation of CHO cells stably transfected with the chick M₃ receptor resulted in a marked pertussis toxin-insensitive stimulation of inositol phosphate release. Muscarinic stimulation was also coupled to a 1.9-fold increase in cAMP levels. Both of these responses have been shown to be characteristic of M₃ coupled receptors (Peralta et al., 1988).

Previous reports from Tietje et al. (1990) and Tietje and Nathanson (1991) demonstrated that chick heart was unique in that it expressed mRNAs coding for both the M₃ and M₄ muscarinic receptor isoforms. The finding that mRNA coding for an M₃ muscarinic receptor expressed in chick heart is also unique for chick and has not been reported in hearts of other species. One possibility for the finding that chick heart expresses multiple muscarinic receptor isoforms is that in the chick the coupling of inositol phosphate production to muscarinic stimulation may be different than in cardiac tissues from other species. Barnett et al. (1990) and Stephan et al. (1991) demonstrated that early in embryonic development at day 5 in ovo, muscarinic stimulation of IP₃ and diacylglycerol production in cultured chick atrial cells appears to be totally insensitive to pertussis toxin inhibition. Between days 5 and 14 in ovo muscarinic stimulation of IP₃ formation became partially sensitive to pertussis toxin inhibition. We have demonstrated that embryonic chick heart expresses an M₃ muscarinic receptor mRNA which, when expressed in CHO cells, codes for a receptor that couples to PLC via a pertussis toxin-insensitive G-protein and contains an amino acid sequence which has been shown in other species to be specific for binding to a pertussis toxin-insensitive G-protein. These data support the conclusion that early in embryonic development an M₃ muscarinic receptor is coupled to IP₃ and DG production via only a pertussis toxin-insensitive G-protein and that subsequently, after day 5 in ovo, other muscarinic receptor subtypes such as M₂ and M₄ become coupled to PLC via both a pertussis toxin insensitive and newly appearing pertussis toxin-sensitive G-proteins. Thus during embryonic development a switch in the subtype of muscarinic receptor or G-proteins which couple muscarinic stimulation to PLC activity might explain developmental changes in receptor-effector coupling.

REFERENCES