Characterization of Muscarinic Acetylcholine Receptors Expressed by an Atrial Cell Line Derived From a Transgenic Mouse Tumor

Mary E. Morton, Craig Brumwell, Cindy L. Gartside, Stephen D. Hauschka, Neil M. Nathanson

Abstract The properties of muscarinic acetylcholine receptors in the cell line MCM1, derived from an SV40 T-antigen-induced atrial tumor in a transgenic mouse, were determined. Binding studies using the nonselective muscarinic antagonist [(3)H]quinuclidinyl benzilate, the M1-selective antagonist pirenzepine, and the M2-selective antagonist AFDX-116 indicate that the receptors have the pharmacological properties of the cardiac (M2) receptor subtype. The receptors could be immunoprecipitated with a monoclonal antibody specific for the cardiac receptor, thus confirming the identity of the receptors expressed in these cells. The types of G proteins expressed in the cells were determined by Northern blot analyses: mRNA encoding the α subunits of G1a, G1b, and G1c, but not G1α, or G1α, were detected, consistent with previous observations of neonatal mammalian atria. The muscarinic receptors were functionally active, as demonstrated by the ability of the agonist to stimulate phosphoinositide turnover and to inhibit adenylyl cyclase activity. The availability of a mammalian atrial cell line that continues to express the appropriate functionally coupled subtype of muscarinic receptor may provide a useful system for the investigation of the regulation of expression and function of cardiac muscarinic receptors. (Circ Res. 1994;74:752-756.)

Key Words • G proteins • phospholipase C • adenylyl cyclase • cardiac cell cultures • SV40 T antigen

Stimulation of the parasympathetic ganglia causes the release of acetylcholine, which acts on muscarinic acetylcholine receptors (mACHRs) on the myocardial cell surface. Activation of the mACHRs regulates a variety of physiological, biochemical, and electrophysiological responses: decrease in rate (negative chronotropic effect) and force (negative inotropic effect) of contraction, inhibition of adenylate cyclase activity, stimulation of phospholipase C and A2 activities, activation of an inward-rectifying potassium channel, inhibition of a calcium channel, and inhibition of the hyperpolarization-activated pacemaker current. All of these responses are mediated, either directly or indirectly, via the action of one or more GTP-binding regulatory proteins, or G proteins (see References 1 and 2 for reviews). Investigation of the mechanisms of action and regulation of mACHRs in the heart has been greatly aided by the ability to study the mACHR on cardiac cells in culture.1 Although the use of primary cells has provided a wealth of information on the biochemical and physiological properties of the receptor, many types of studies (eg, gene transfection and stable expression) could be performed either more conveniently or only with a continuous cell line. Attempts to isolate continuous cardiac muscle cell lines using techniques such as repeated serial passage of cells have not been successful—the resulting cell lines no longer retain the properties expected of cardiac cells (eg, see Reference 3).

Received May 4, 1992; accepted December 16, 1993.

From the Departments of Pharmacology (M.E.M., C.B., N.M.N.) and Biochemistry (C.L.G., S.D.H.), University of Washington, Seattle; and the Department of Biology (M.E.M.), College of the Holy Cross, Worcester, Mass.

Correspondence to Dr Neil M. Nathanson, Department of Pharmacology, SJ-30, University of Washington, Seattle, WA 98195.

It has been recently shown that the expression of the v-my c oncogene in chick hearts4 and of the SV40 T antigen in the hearts of transgenic mice5,6 induces the formation of cardiac tumors, and the expression of SV40 T antigen in cultured rat heart cells permits maintained proliferation of differentiated myocardial cells.7 Behringer et al8 reported that expression of the SV40 T antigen in the hearts of transgenic mice resulted in the production of rhabdomyosarcoma tumors in the right atrium; cells cultured from these tumors retained many of the properties of differentiated cardiac cells such as continued expression of contractile proteins. Steinhelper et al9 also found that cells cultured from such tumors continued to express ultrastructural and contractile properties expected of cardiac cells. Sculp- toreanu et al10 recently reported that the MCM1 cell line used in our studies expresses a cAMP-regulated and β-adrenergic receptor-regulated tetrodotoxin-insensi- tive sodium channel that is similar to the embryonic type of channel found in other cardiac and skeletal muscle preparations. In the present study, we show that the MCM1 cells also continue to express the subtypes of muscarinic receptors and G proteins expected in mammalian cardiac cells.

Materials and Methods

Cell Culture

The MCM1 cell line was obtained from cells derived from an atrial tumor that arose after the expression of the SV40 T antigen in a transgenic mouse.3 The cells were cultured as previously described10 in Ham’s F-10 medium with the following additions: 15% horse serum, 0.06 mg/mL gentamicin, and 0.96 mmol/L additional CaCl2. Briefly, confluent cells were passaged by trypsinization and plated at one half the preceding density on gelatin-coated (0.5%) tissue culture–treated plates. Cells were fed every 3 days and were split when
confluent (usually every 10 days). Approximately 25% of the cells in the culture continued to express myosin heavy chain as determined by immunocytochemistry.5

**Ligand-Binding Assays**

The binding of the muscarinic radioligand [3H]quinuclidinyl benzilate ([3H]QNB, Amersham, 41 Ci/mmol) to the mAChR in membranes homogenates and ligand/[3H]QNB competition assays were performed and analyzed as previously described.11,12 In brief, cells were homogenized in 50 mmol/L NaPO4, pH 7.4, and membranes were pelleted and washed twice by centrifugation before resuspension in 50 mmol/L NaPO4, pH 7.4. Binding assays contained 50 to 100 μg of protein and were incubated for 90 minutes for saturation binding analyses and 60 minutes for competition experiments. Nonspecific binding was defined as the amount of binding observed in the presence of 1 μmol/L atropine and was generally 5% to 15% of total binding.

**RNA Isolation and Northern Blot Analysis**

Total cellular RNA was isolated by homogenization in guanidinium isothiocyanate and centrifugation in cesium chloride as described by Maniatis et al.13 RNA was subsequently size-fractionated under denaturing conditions on agarose-formaldehyde gels and hybridized to cDNA probes for G protein α and β subunits as described by Uhler et al14 and Luetje et al.15

The G-protein cDNA probes were obtained as follows: a 0.7-kb EcoRI fragment isolated from a full-length rat Gαα cDNA clone, a full-length cDNA clone for rat Gαβ1, and a near-full-length clone for Gαβ2 were obtained from Drs H. Itoh and Y. Kaziro16; a 0.6-kb XbaI-EcoRI fragment was isolated from a full-length clone for rat Gαα, and a 0.4-kb BamHI fragment was isolated from a full-length clone for rat Gαβ1 (full-length Gαα) and Gαβ3 clones were obtained from Dr R. Reed17; and a full-length cDNA clone for bovine Gβ1 was obtained from Drs J. Hurley and M. Simon.18

After denaturing in buffer containing 2.2 mol/L formaldehyde and 50% formamide, the samples were subjected to electrophoresis on a 1% agarose gel, transferred to Nytran nylon membranes, baked, and hybridized under conditions of moderately high stringency to the 32P-labeled nick-translated probes (106 cpm/mL). The hybridization conditions were as follows: 50% formamide, 6× standard saline citrate (SSC) (1× SSC contains 0.15 mol/L NaCl and 0.015 mol/L sodium citrate), 50 mmol/L NaHPO4 (pH 7.4), 5.0 mmol/L EDTA, 0.1% sodium dodecyl sulfate (SDS), 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1% Ficoll, and 100 μg/mL salmon sperm DNA, at 37°C. The washing conditions were empirically determined to minimize background while yielding strong signals with the rat and bovine probes used here. All filters were washed in 2× SSC, 0.5% SDS, and 0.1% sodium pyrophosphate at room temperature. Filters were subsequently washed as follows: Gαα, Gαβ1, Gαβ2, and Gαβ3, 0.5× SSC, 0.5% SDS, and 0.1% sodium pyrophosphate, 37°C; Gαα, 0.5× SSC, 0.5% SDS, and 0.1% sodium pyrophosphate, room temperature; and Gβ1, 2× SSC, 0.5% SDS, and 0.1% sodium pyrophosphate, 52°C.

**Phosphoinositide Hydrolysis Assays**

Cells were grown on 35-mm tissue culture plates and incubated overnight with culture medium containing 1 μCi/mL [3H]myo-inositol (18.9 Ci/mmol, Amersham Corp). Carbachol-mediated stimulation of phosphoinositide turnover was measured by the method of Masters et al19 as described by Subers and Nathanson.20 All experiments were performed in triplicate.

**Immunoprecipitation Analysis of mAChR**

Immunoprecipitation analyses were carried out as described by Luetje et al.21 In brief, the mAChR was solubilized with digitonin-cholate (1.0%–0.1%) and incubated with Immunobeads (BioRad) precoated with monoclonal antibody 31-1D1 specific for the cardiac mAChR. After centrifugation, the amount of mAChR precipitated was determined by the binding of [3H]QNB.

**Adenylate Cyclase Assays**

Cells were grown on 100-mm tissue culture plates; after homogenization and preparation of membranes, adenyl cyclase activity was determined as described previously.22

**Results**

To determine whether MCM1 cells express mAChR, ligand-binding analysis with the mAChR antagonist [3H]QNB was carried out. [3H]QNB was bound in an atropine-displaceable saturable fashion to MCM1 membranes (Fig 1, left). Scatchard analysis (Fig 1, left, inset) indicated that [3H]QNB was bound to a homogeneous class of high-affinity sites with a Kd of 47±5 pmol/L (n=4, mean±SEM). This is similar to the affinity of [3H]QNB for mAChR in membranes prepared from mammalian hearts. The density of mAChR binding sites, 500 to 600 fmol/mg protein, was also similar to that found in cardiac membranes.11

To determine the pharmacological binding properties of the mAChR expressed in MCM1 membranes, the affinities for the subtype-selective antagonists pirenzepine and AFDX-11623 were determined (Fig 1, right). The binding of both ligands was well described by their interaction with a single class of binding sites, with Hill coefficients of 0.96±0.13 and 0.95±0.09, respectively (n=3, mean±SD). The Kd for pirenzepine was 6.28+1.78×10−8 mol/L, similar to the relatively low affinity characteristic of the cardiac mAChR.

Our laboratory previously described the isolation and characterization of a number of monoclonal antibodies raised against the purified porcine atrial mAChR.21 These antibodies recognize the mAChR solubilized from the hearts of diverse mammalian species, and they exhibit a high degree of specificity for the M2 (cardiac) subtype of receptor. Therefore, we used monoclonal antibody 31-1D1 to confirm that the mAChR expressed in MCM1 cells corresponded to the M2 cardiac receptor subtype. As shown in Fig 2, the antibody was able to precipitate approximately 80% of the mAChR solubilized both from cardiac membranes and from MCM1 cell membranes. Thus, the mAChR expressed in MCM1 cells exhibits the pharmacological and immunological properties expected of the cardiac mAChR subtype.

Muscarinic receptors mediate physiological responses by interacting with the G proteins. Previous studies15 using nucleic acid probes demonstrated that there are tissue-specific and developmental changes in the expression of G proteins in the mammalian heart. Northern blot analyses were carried out to determine the types of G proteins expressed in MCM1 cells (Fig 3). The mRNAs for Gαα, Gαβ1, Gαβ2, and Gβ were readily observed, whereas mRNAs for Gαβ3 and Gαβ4 were not detected. This pattern of expression is similar to that seen in neonatal atria and differs from that seen in adult atria and neonatal and adult ventricles.15 Thus, the pattern of G-protein expression in
MCM1 cells is that expected for atrial cells at a relatively early stage of development.

To test that the inhibitory G proteins (Gi proteins) were functionally active, the ability of the nonhydrolyzable GTP analogue GppNHp to inhibit forskolin-stimulated adenylate cyclase activity was determined. Scammon and Daly\(^{24}\) demonstrated that at short incubation times low concentrations of GppNHp could activate Gi proteins to mediate receptor-independent inhibition of adenylate cyclase activity. As shown in Fig 4, left, GppNHp caused a significant decrease (40% to 45%) in forskolin-stimulated adenylate cyclase activity in membranes prepared from MCM1 cells. Both the extent of inhibition and the concentration dependence for GppNHp are similar to those reported previously for cardiac cells.\(^{22}\)

Cardiac muscarinic receptors mediate a number of physiological responses, including inhibition of adenylate cyclase activity, stimulation of phospholipase C and guanylate cyclase activities, activation of inwardly rectifying potassium channels, and inhibition of calcium channels and the pacemaker current. As a first step in determining if the mAChR in MCM1 cells is physiolog-
ically active, we examined the ability of muscarinic agonists to stimulate phosphoinositide hydrolysis. Carbachol caused a significant increase in phosphoinositide hydrolysis (Fig 4, middle); this stimulation was blocked by the muscarinic antagonist atropine. The magnitude of this response is similar to that seen in primary cultured cardiac cells.\(^{20}\) We also determined if the mAChR could inhibit adenylyl cyclase activity in membranes homogenates. Consistent with previous results,\(^ {22}\) carbachol caused approximately a 30% inhibition of adenylyl cyclase activity (Fig 4, right). Thus, the mAChRs expressed in MCM1 cells are functionally active.

**Discussion**

These results demonstrate that MCM1 cells express a functionally active mAChR that by pharmacological and immunological criteria is the subtype normally found in cardiac cells. In addition, the cell line expresses the pattern of G-protein genes normally found in neonatal atrial cells. Although the MCM1 cells have a relatively slow growth rate, they do provide a source of large numbers of homogenous cultures. The availability of a mammalian cardiac cell line expressing the appropriate mAChRs, \(\beta\)-adrenergic receptors, G proteins, and ion channels\(^ 8\) should prove valuable for a variety of molecular, biologic, biochemical, and physiological studies on the regulation and function of signal transduction processes in cardiac cells.

**Acknowledgments**

This research was supported by grants HL-44948 and HL-39070 from the National Institutes of Health (NIH), by a Grant-in-Aid from the American Heart Association, and by an NIH postdoctoral fellowship to Dr Morton.

**References**


Characterization of muscarinic acetylcholine receptors expressed by an atrial cell line derived from a transgenic mouse tumor.

M E Morton, C Brumwell, C L Gartside, S D Hauschka and N M Nathanson

_Circ Res._ 1994;74:752-756
doi: 10.1161/01.RES.74.4.752

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1994 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/74/4/752

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/