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Expression of a Cloned Muscarinic Receptor in A9 L Cells

MARK R. BRANN, NOEL J. BUCKLEY, S. V. PENELOPE JONES, and TOM I. BONNER

Metabolic Diseases Branch, National Institute of Diabetes, Digestive and Kidney Diseases (M.R.B.), Laboratory of Neurophysiology, National Institute of Neurological and Communicative Disorders and Stroke (S.V.P.J.), and Laboratory of Cell Biology, National Institute of Mental Health (N.J.B., T.I.B.), Bethesda, Maryland 20892

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SUMMARY

Using an oligonucleotide based on the sequence of a porcine brain muscarinic receptor cDNA, we recently cloned four distinct muscarinic receptors from the rat and human genomes. In the present study we transfected the rat homolog of the porcine brain muscarinic receptor cDNA into A9 L cells using a mammalian expression vector and a calcium phosphate precipitation procedure. Before transfection, A9 L cells do not bind muscarinic ligands and do not express muscarinic receptor mRNA. After transfection, A9 L cells expressed muscarinic receptor mRNA and saturable, high affinity binding sites for the muscarinic antagonists ^{3}H-quinuclidinyl benzilate and ^{3}H-pirenzepine. The muscarinic receptor antagonists AF DX-116 and pirenzepine displaced bound ^{3}H-quinuclidinyl benzilate with inhibition curves suggestive of a single high affinity binding site. Competition of ^{3}H-quinuclidinyl benzilate-labeled sites with the agonists acetylcholine and carbachol yielded broad inhibition curves, consistent with a heterogeneity of binding sites. In the presence of guanine nucleotide, the agonist inhibition curves were steeper, suggesting the presence of a single low affinity site. The effects of guanine nucleotides on agonist binding are consistent with coupling of these receptors to a guanine nucleotide-binding protein (G-protein) endogenous to A9 L cells. The electrical properties of the transfected A9 L cells were examined using the whole cell patch-clamp technique. Fifty μM acetylcholine induced a conductance which reversed in polarity at −60 mV. This conductance could be reversibly blocked by atropine. These data illustrate the utility of stable transfection of A9 L cells for the characterization of individual cloned muscarinic receptors, their G-protein coupling mechanisms, and resultant physiological responses.

Muscarinic receptors have been divided into two classes based on the selectivity of the muscarinic antagonist PZP: M1 receptors have high affinity for PZP and are abundant in cerebral cortex, and M2 receptors have low affinity for PZP and are abundant in heart (1). Many agonists are also selective for muscarinic receptor subtypes. However, the use of agonists for the characterization of these sites is complicated by the ability of agonists to discriminate multiple affinity states of receptors. For example, the agonists ACH and carbachol bind with higher affinity to muscarinic receptors which are coupled to G-protein than to uncoupled receptors (2–4).

Activation of muscarinic receptors elicits a number of responses including activation of a soluble guanylate cyclase and stimulation of a high affinity GTPase, phospholipid turnover, and cAMP phosphodiesterase activity; inhibition of adenylate cyclase; and opening of potassium and chloride channels (5–7). The GTPase is intrinsic to the G-proteins to which muscarinic receptors couple. Indirect evidence suggests that the activation of cAMP phosphodiesterase and guanylate cyclase activities may at least in part be mediated by the stimulation of phospholipid turnover (5, 6). Recent evidence indicates that muscarinic receptors are able to couple with several distinct G-proteins and that different G-proteins may mediate the molecular responses to receptor activation. For example, in phospholipid vesicles, purified muscarinic receptors couple to both G_i and G_o (3, 4). G_i and G_o are major G-proteins of brain which are inactivated by ADP-ribosylation catalyzed by pertussis toxin. Inhibition of adenylate cyclase and opening of potassium channels by muscarinic receptors are also blocked by pertussis toxin, whereas stimulation of phospholipid turnover is not, suggesting that these responses are mediated by distinct G-proteins (5, 6). The precise identity of these G-proteins is unknown.

Unlike many other receptor-mediated events, e.g., stimulation and inhibition of adenylate cyclase by dopamine D1 and D2 receptors, respectively (8), the molecular and physiological responses to muscarinic receptor activation and their concomitant G-protein-mediated coupling mechanisms have not been clearly linked to pharmacologically defined receptor subclasses. The failure to obtain a unified classification of muscarinic receptor subtypes may in part be due to the limited selectivity of the available muscarinic ligands, and the heterogeneity of muscarinic receptors present in most tissues.

ABBREVIATIONS: PZP, pirenzepine; ACH, acetylcholine; G_i, inhibitory guanine nucleotide-regulatory protein; G_o, guanine nucleotide-regulatory protein of unknown function; QNB, quinuclidinyl benzilate; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; I-V, current-voltage.

The recent cloning of two porcine muscarinic receptor cDNAs, one from heart (9) and one from brain (10), provides a new tool for the characterization of multiple muscarinic receptors and their coupling mechanisms. Based on the cDNA sequence of the brain muscarinic receptor, we recently cloned four distinct muscarinic receptors from a rat cDNA and rat and human genomic libraries. Two of these four receptors correspond to the porcine brain and heart cDNAs, while the other two are expressed preferentially in brain and have high affinity for muscarinic ligands when transiently expressed in COS-7 cells (11). In the present study we have transfected cDNA encoding the rat homolog of the porcine brain muscarinic receptor into A9 L cells. This receptor has 98% amino acid identity with the porcine brain receptor. We have refered to this receptor as the "m1" receptor to distinguish it from the other two brain receptors, "m3" and "m4" (11). A9 L cells stably express this cloned muscarinic receptor. We have characterized the pharmacological properties of the cloned receptor and tested its ability to couple to G-proteins endogenous to the A9 L cells. Finally, we examined the electrical events related to activation of the cloned muscarinic receptor.

Materials and Methods

Isolation of muscarinic receptor cDNA. A rat muscarinic receptor abundant in cerebral cortex was cloned from a plasmid cerebral cortex cDNA library in the pcD mammalian expression vector as described elsewhere (11). Briefly, a 56 base oligodeoxynucleotide complementary to bases 170–225 of the porcine muscarinic receptor cDNA (10) was synthesized and used as a hybridization probe. The plasmid DNA containing the rat cDNA was purified by a lysozyme-sodium dodecyl sulfate procedure followed by two cycles of banding on CsCl density gradients (12). Since the cDNA library was constructed with a mammalian expression vector (13), the isolated cDNA was directly transfected into A9 L cells.

A9 L cell transfection. Murine A9 L cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and plated at a density of 5×10^5 cells/10-cm Petri dish 24 hr prior to transfection. Eighteen μg of plasmid DNA were mixed with 2 μg pcDneo (the pcD vector expressing a neomycin resistance gene, H. Okayama) and transfected into A9 L cells by calcium phosphate precipitation (14). The growth medium was changed 24 hr after transfection. Selection was begun 48 hr after transfection with the neomycin analog G418 (400 μg/ml). Selection was continued for 2 weeks, and the growth medium and drug were changed every 3 days. Binding assays were performed a minimum of 1 week after G418 was removed from the growth medium.

Radioligand binding assays. ^{3}H-QNB (33 Ci/mmol) and ^{3}H-PZP (85 Ci/mmol) were obtained from New England Nuclear; PZP and AF DX-116 were from R. Hammer, Boeringer Ingelheim, West Germany; and atropine sulphate, ACH chloride, carbachol, and oxotremorine were from Sigma. A9 L cells were scraped from flasks after addition of 10 ml of PBS, pH 7.4, and collected by centrifugation at $2,000 \times g$. The cells were resuspended in 10 mM HEPES, 5 mM $MgCl_2$, pH 7.4, and homogenized in a tissue disruptor (Brinkman). Membranes were pelleted at $15,000 \times g$ and resuspended in HEPES buffer at a protein concentration of ~0.05 mg in 400 μl. Binding assays were initiated by addition of 400 μl of membranes to 100 μl of ligands. Saturation experiments were performed in a final volume of 1 ml. Membranes were incubated for 2 hr at room temperature. Nonspecific binding was defined with 1 μM atropine.

Analysis of binding data. The radioligand binding data were analyzed by nonlinear regression using the program DATAPLOT (distributed by the National Technical Information service) run on a VAX II computer. The experimental data from saturation experiments were fit to the function $y = ax^N/k/(1 + x^N/k)$, where y is specific radioligand bound, x is free ligand concentration, N is Hill number, k is k_D, and a is the B_{max}. The experimental data from the inhibition experiments were fit to the function $y = 100 - 100\ x^N/k/(1 + x^N/k)$,where y is percentage inhibition of labeled sites, N is Hill number, and k is IC_{50}.

Visualization of muscarinic receptor mRNA. Muscarinic receptor mRNA was measured in transfected and non-transfected cells using modifications of *in situ* hybridization procedures which we have previously applied to the measurement of neuropeptide (15) and G-protein (16) mRNAs. A synthetic oligonucleotide probe complementary to the 48 bases (5′-TGG TGC CAA GAC AGT GAT GTT GGG ACT GAC AGC AGG GGG CAC TGA GGT-3′) which encode the amino terminus, amino acids 3–18, of the rat muscarinic receptor was made on an Applied Biosystems DNA synthesizer and purified on a preparative sequencing gel. This sequence differs substantially from the related muscarinic receptor sequences which we have cloned. The purified probe was labeled by tailing the 3′ end with [α-^{32}P]deoxyadenosine triphosphate (>3000 Ci/mmol, New England Nuclear) and terminal transferase (Bethesda Research Laboratory). A9 L cells simultaneously transfected with muscarinic receptor cDNA and pcDneo, and control cells transfected with pcDneo without muscarinic cDNA were grown on cell culture slides (Miles Scientific). After 24 hr, the cells were rinsed in PBS, fixed in 4% PBS formaldehyde for 10 min, rinsed in PBS, and dried. Slides were stored at −70° until used for hybridizations. Slides were thawed and incubated in 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl (pH 8) for 10 min at room temperature. Slides were transferred through 70 (1 min), 80 (1 min), 95 (2 min), 100 (5 min), and 95% (1 min) ethanol and air-dried. Hybridizations were performed in 4× SSC, 50% formamide, 1× Denhardt's, 250 μg/ml yeast tRNA, 500 μg/ml sheared single-stranded salmon sperm DNA, and 10% dextran sulfate. One million dpm of oligonucleotide probe were applied in 25 μl of hybridization buffer to the cells in each culture chamber. Slides were covered with a parafilm coverslip and incubated overnight at 37° in a humid chamber. Slides were washed in 1× SSC at 55°, rinsed in H_2O, and air dried. Slides were exposed to X-ray film for 4 days with one intensification screen (Cornex, Picker).

Electrophysiological recordings. A9 L cells previously transfected with muscarinic receptor cDNA and non-transfected A9 L cells were maintained at 37° in 3-cm culture dishes for 4–7 days. Electrical recordings were made on an inverted phase-contrast microscope at room temperature (~22°). The cells were bathed in a solution containing (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES (pH 7.4). Membrane currents were recorded using the whole cell patch-clamp technique (List EPC-7) (17). Patch pipettes (4–7 MΩ) were filled with a solution containing (in mM): 150 potassium gluconate, 2 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA, 5 ATP (Mg salt), 0.5 GTP (Li salt), 5 HEPES (pH 7.2). Cells were voltage-clamped at −50mV and the currents induced by ACH were recorded. ACH (50 μM) was dissolved in the bathing medium and applied by pressure (1–2 p.s.i.) from a micropipette positioned 5–10 μm from the cell. Atropine was applied directly to the bathing medium.

Results

Fig. 1 illustrates the *in situ* hybridization of our oligodeoxynucleotide probe to muscarinic receptor mRNA in A9 L cells transfected with pcDneo without muscarinic receptor cDNA, and pcDneo with the muscarinic receptor cDNA. Only cells transfected with muscarinic receptor cDNA expressed mRNA detectable with the oligonucleotide complementary to muscarinic receptor mRNA. Although we have not quantitated the levels of mRNA which these hybridization signals represent, more mRNA seems to be present in these cells than is endogenously expressed in brain and neuronal cell lines when observed using similar methods.[1] (unpublished observations).

Fig. 2 illustrates the binding of the muscarinic receptor

[1] M. R. Brann, N. J. Buckley, and T. I. Bonner, unpublished observations.

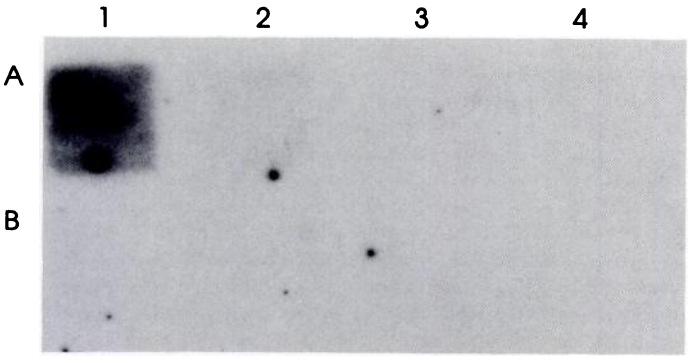

Fig. 1. *In situ* hybridization of muscarinic receptor mRNA in transfected A9 L cells. A total 1×10^6 (*A1, B1, A3, B3*) and 1×10^5 (*A2, B2, A4, B4*) A9 L cells (*A*) transfected with pcDneo and muscarinic receptor cDNA and (*B*) transfected with pcDneo without muscarinic receptor cDNA were grown for 24 hr in wells of tissue culture slides. The first slide (*1, 2*) was hybridized with the muscarinic receptor oligonucleotide probe, and the control slide (*3, 4*) was hybridized with another oligonucleotide probe to the amino terminal of one of the related muscarinic receptors we have cloned from rat cerebral cortex. This sequence is not present in the muscarinic receptor cDNA used here or the pcD vector, nor is it endogenous to A9 L cell mRNA.

antagonists ^{3}H-QNB and ^{3}H-PZP to membranes prepared from A9 L cells transfected with muscarinic receptor mRNA. In similar experiments with non-transfected A9 L cells and A9 L cells transfected with pcDneo alone, no specific binding of either ligand was observed (not shown). After transfection with muscarinic receptor cDNA and pcDneo, saturable binding sites were observed for both ligands and the following parameters were observed: a B_{max} of 194 ± 11 fmol/mg of protein and a k_D of 59 ± 25 pM for ^{3}H-QNB, and a B_{max} of 129 ± 24 fmol/mg of protein and a k_d of 14 ± 9 nM for ^{3}H-PZP. The significance of the small difference in the B_{max} values of these two ligands is unclear. Such a small difference may be related to imprecise knowledge of absolute specific activity or radioligand stability.

Competition of sites labeled with ^{3}H-QNB by the muscarinic antagonists AF DX-116 and PZP is illustrated in Fig. 3. Both of these ligands displaced all of the labeled muscarinic receptors with high affinity and the following parameters were observed: an IC_{50} of 5.6 ± 0.7 nM for PZP and 6.4 ± 1.1 μM for AF DX-116. Both of the inhibition curves were steep, with Hill numbers (0.96 ± 0.06 for PZP and 0.90 ± 0.07 for AF DX-116) close to unity. Competition of sites labeled with ^{3}H-QNB by the agonists carbachol, ACH, and oxotremorine are illustrated in Fig. 4. The best fit parameters of these inhibition curves are presented in Table 1. Each of these ligands displaced all of the ^{3}H-QNB-labeled sites with high affinity. The inhibition curves for carbachol and ACH were broad, with Hill numbers well below 1. When the membranes were incubated with 300 μM GTP, the agonist inhibition curves were shifted to the right, the curves were steeper, and the Hill numbers increased toward 1. Relatively little effect of GTP was observed on the binding of oxotremorine and no effect of GTP was observed on the direct binding of ^{3}H-QNB (not shown).

The electrical properties of A9 L cells transfected with muscarinic receptor cDNA were examined using the whole cell patch-clamp technique. The cells had a resting membrane potential of approximately −65 mV and were electrically inexcitable. Application of ACH to the cells produced an outward current when voltage-clamped at −50 mV. A typical response is shown in Fig. 5A. Development of the outward current

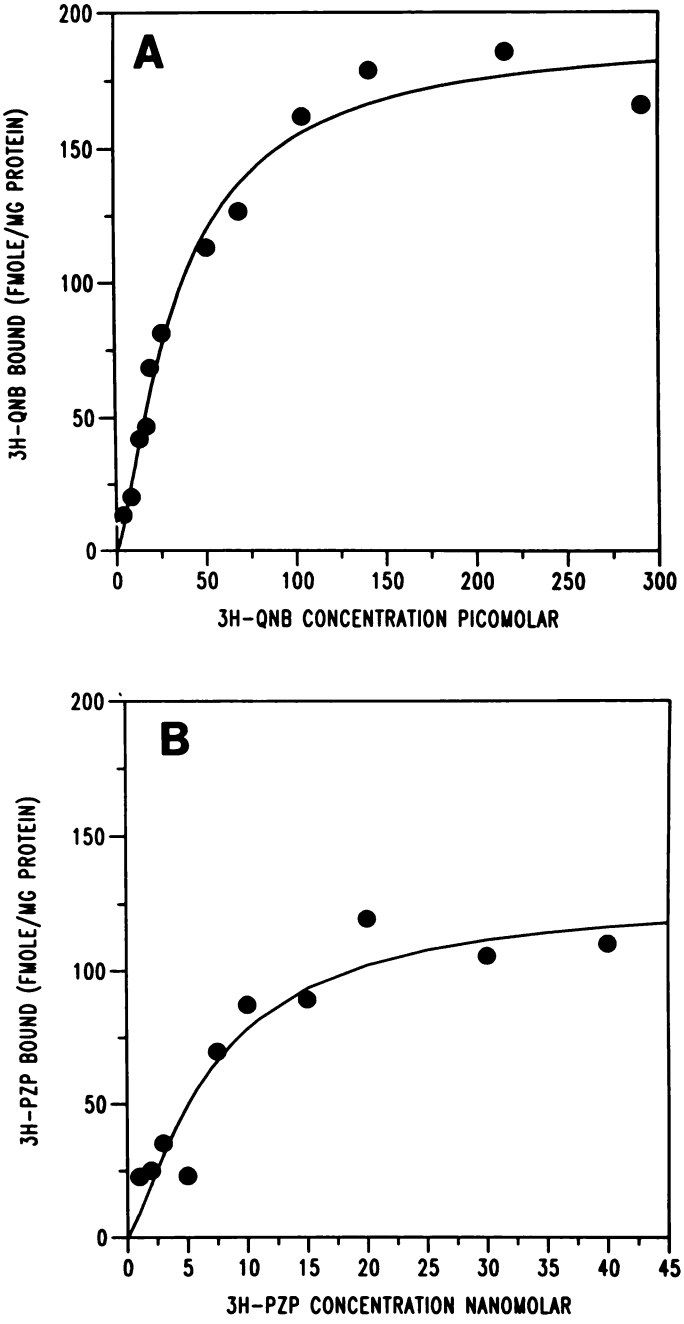

Fig. 2. Specific binding of ^{3}H-QNB and ^{3}H-PZP to A9 L cell membranes. A9 L cells were simultaneously transfected with pcDneo and muscarinic receptor cDNA. Increasing concentrations of ^{3}H-QNB and ^{3}H-PZP (*B*) were incubated with 50 μg of A9 L cell membranes in 500 μl of buffer. Nonspecific binding was defined with 1 μM atropine. *Lines* are nonlinear least squares fits of the data to a single mass-action binding site with a variable Hill number. Identical experiments were simultaneously performed with A9 L cell membranes from cells transfected with pcDneo without muscarinic receptor cDNA. In these cells no specific binding of ^{3}H-PZP or ^{3}H-QNB was observed.

showed a considerable delay after the onset of the ACH pulse, in this example by about 3 sec. The response to ACH was abolished by 1 μM atropine applied to the bathing solution (Fig. 5B) and was restored after washout (Fig. 5C). Tubocurarine chloride (50 μM) had no effect on the ACH responses. Control cells (non-transfected) did not produce a response to ACH. Fig. 5D shows the peak current-voltage (I-V) relation of the ACH

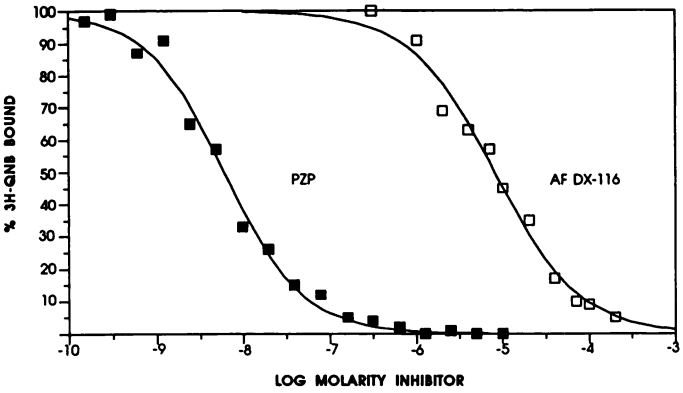

Fig. 3. Displacement of ^{3}H-QNB-labeled sites by the muscarinic antagonists PZP and AF DIX-116. Membranes were prepared from A9 L cells transfected simultaneously with pcDneo and muscarinic receptor cDNA. Sites were labeled with 100 pM ^{3}H-QNB and nonspecific binding was defined with 1 μM atropine. Lines are computer-generated fits of the data to a single mass-action binding site with a variable Hill number.

response, obtained by voltage-clamping the cell at a range of potentials. The reversal potential of the ACH-activated current is approximately −60 mV. This is close to the reversal potential for the chloride ion in these solutions (approximately −76 mV).

Discussion

In agreement with the results of Kubo *et al.* (10), our data illustrate that a single cDNA encodes a protein which binds muscarinic ligands. Use of a stably transfected cell line expressing a pure receptor subtype to characterize receptor expression has many advantages relative to the translation of mRNA in oocytes as used previously. The transfected cell line has the membrane environment of mammalian tissues, allowing a more direct comparison of pharmacological properties of the cloned receptor with endogenously expressed mammalian receptors. Because the cell is relatively homogeneous and has the potential for unlimited expansion, detailed characterization of the cloned receptor and its coupling mechanisms is possible.

The binding sites expressed in the A9 L cells are homogeneous with respect to antagonist binding. Displacement of these sites with agonists suggests a heterogeneity or cooperativity of sites, and agonist binding is sensitive to GTP. Reconstitution experiments have shown that the complexity in agonist binding to muscarinic receptors is, at least in part, induced by the coupling of muscarinic receptors to G-proteins, and that agonists distinguish muscarinic receptors which are coupled from those which are not (2–4). Our data strongly suggest that muscarinic receptors couple with a G-protein or proteins endogenous to the A9 L cell membrane. Using oligonucleotides which we have described elsewhere, we have measured the G-protein mRNAs which A9 L cells express. These cells express G_s and two forms of G_i mRNA, but not G_o or a third form of G_i mRNA;[2] the two latter G-protein mRNAs have thus far only been found in neuronal tissues (18).[3]

The affinity of the binding sites expressed in A9 L cells for the antagonists PZP and AF DX-116 is similar to that reported for M1 muscarinic receptors in brain, consistent with the assignment of this cloned muscarinic receptor to the M1 class

[2] M. R. Brann, N. J. Buckley, R. C. Collins, and A. Spiegel, unpublished observations.

[3] M. R. Brann, N. J. Buckley, and T. I. Bonner, unpublished observations.

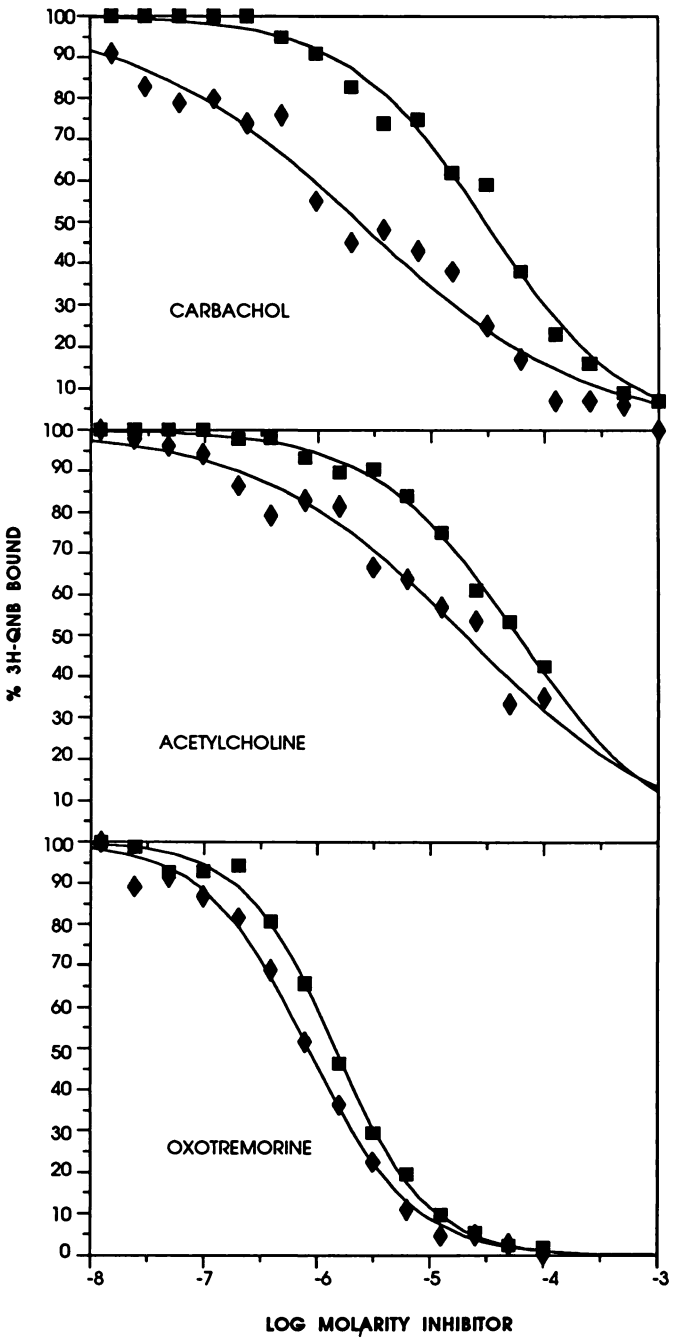

Fig. 4. Influence of GTP on the displacement of ^{3}H-QNB-labeled sites by muscarinic agonists. Membranes were prepared from A9 L cells transfected simultaneously with pcDneo and muscarinic receptor cDNA. Sites were labeled with 100 pM ^{3}H-QNB and nonspecific binding was defined with 1 μM atropine. The indicated agonists were incubated in the absence (♦) and presence (■) of 300 μM GTP. *Lines* are nonlinear least squares fits of the data to a single mass-action site with a variable Hill number.

(1, 6). This assignment is further supported by the tissue distribution of mRNA encoding this receptor. The porcine mRNA homolog is highly enriched in cerebral cortex relative to heart (10), and we have shown using *in situ* hybridization that this mRNA has a pattern of expression within brain similar to that expected of the M1 muscarinic receptors.[3] The properties of agonist binding to the cloned muscarinic receptors are also consistent with their M1 assignment. ACH and carbachol are pure agonists at M1 muscarinic receptors, while

TABLE 1

Best fit parameters of agonist inhibition curves

Values (μM) are nonlinear least squares fits of the data presented in Fig. 4 to a single mass action binding site with a variable Hill number. Several concentrations of the muscarinic agonists were incubated with 100 pM ^{3}H-QNB. Nonspecific binding was defined with 1 μM atropine.

	Control		+300 μM GTP	
	IC_{50}	Hill no.	IC_{50}	Hill no.
Carbachol	0.52 ± 0.03	0.4 ± 0.04	2.2 ± 0.17	0.7 ± 0.04
ACH	4.2 ± 0.36	0.5 ± 0.03	17.0 ± 1.51	0.7 ± 0.03
Oxotremorine	0.8 ± 0.04	0.9 ± 0.05	1.5 ± 0.08	1.1 ± 0.05

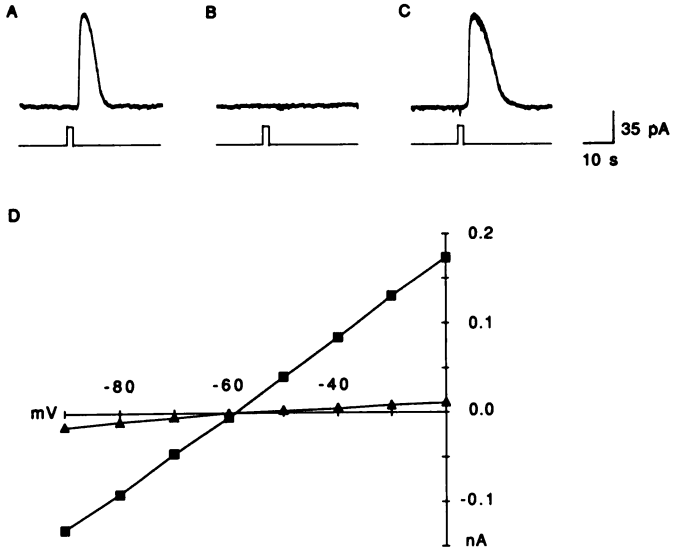

Fig. 5. ACH-induced current responses in A9 L cells transfected with muscarinic receptor cDNA and voltage-clamped at −50 mV. Before, (A), 5 min after addition of 1 μM atropine, (B), and 10 min after wash with new bathing solution (C). *Top traces* show the current responses to 2-sec pulses of ACH (50 μM). An upward deflection denotes outward current. The *lower traces* show the application of ACH. *D.* I-V relationship (inward current shown as negative) in the absence (▲) and presence (■) of ACH (50 μM). Each *point* was obtained from the peak of the response to a 4-sec pulse of ACH at a series of membrane potentials.

oxotremorine is a partial agonist. The efficacies of these agonists may be related to the effects of guanine nucleotides on their binding. That is, at M1 sites in cerebral cortex, ACH and carbachol are highly sensitive to guanine nucleotides, whereas oxotremorine is not. At M2 sites in the heart, oxotremorine is a pure agonist, and its binding is highly sensitive to guanine nucleotides (6).

The electrophysiological data indicate that the cloned muscarinic receptors are able to couple to channels in the A9 L cell membrane. The reversal potential of the ACH-induced conductance indicates that it may be due to the activation of a chloride-conducting channel and the delay in onset of this response suggests the involvement of a second messenger. Similarly, in oocytes the porcine homolog of our cloned receptor couples to a chloride conductance (10). Thus, in oocytes and A9 L cells, which are markedly different cell types, similar cloned muscarinic receptors couple to similar channels. Endogenously expressed muscarinic receptors have also been shown to couple to chloride channels in rat lacrimal glands (7).

The above data illustrate a strategy which can be extended to the analysis of the pharmacological and functional diversity which exists for muscarinic receptors. First, it should be possible to transfect A9 L cells with other muscarinic receptor cDNAs and compare their pharmacology with those described here. Second, since A9 L cells possess a limited repertoire of G-proteins, it will be interesting to examine whether all muscarinic receptors can couple to G-proteins endogenous to A9 L cells. If receptors are identified which are unable to couple with these G-proteins, cDNAs which encode other G-proteins such as G_o and the major G_i of brain could be co-transfected with specific muscarinic receptor cDNAs. Alternatively, other cells that have different endogenous G-proteins may be transfected with muscarinic receptor cDNAs. Finally, if the transfected cells possess the requisite G-proteins, effector enzymes, and channels, the physiological responses mediated by individual cloned muscarinic receptors may be examined in detail.

Acknowledgments

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Note in added proof. While this paper was in review, two relevant reports on cloned muscarinic receptors were published. Peralta *et al.* (*Science Wash. D.C.* **236:** 600, 1987) reported the stable expression of a muscarinic receptor cloned from porcine heart. Their receptor has lower affinity for pirenzepine than does the rat receptor report here, lending further proof of a structural basis of the M1-M2 classification. Fukuda *et al.* (*Nature Lond.* **327:** 623, 1987) reported the expression of both their heart and brain muscarinic receptor clones in oocytes, showing low and high affinity for pirenzepine, respectively, and that the M1, but not the M2 couples to a conductance similar to that reported here.

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Send reprint requests to: Dr. Mark R. Brann, Metabolic Diseases Branch, National Institute of Diabetes, Digestive and Kidney Diseases, Building 10, Room 9C-101, Bethesda, MD 20892.