MOLECULAR PHARMACOLOGY, 26:395–404

Muscarinic Cholinergic Receptors of Two Cell Lines That Regulate Cyclic AMP Metabolism by Different Molecular Mechanisms

TONY EVANS, MCHARDY M. SMITH, LAURA I. TANNER, AND T. KENDALL HARDEN[1]

Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514

Received December 30, 1983; Accepted July 16, 1984

SUMMARY

The attenuation of cyclic AMP accumulation occurs by different mechanisms in 1321N1 astrocytoma cells and NG108-15 neuroblastoma × glioma cells. In 1321N1 cells, cholinergic agonists reduce cyclic AMP accumulation through a Ca^{2+}-dependent activation of phosphodiesterase; in NG108-15 cells, muscarinic receptor-mediated effects on cyclic AMP metabolism occur through inhibition of adenylate cyclase. The goal of the current study was to determine whether different pharmacological specificities were expressed by the muscarinic receptor populations of these two cell lines. The affinity of muscarinic receptors for [^{3}H]quinuclidinyl benzilate (6 pM), [^{3}H]*N*-methylscopolamine (50 pM), and atropine (80 pM) was similar in membrane preparations from each cell line. The affinity of the antagonist, pirenzepine, which has been proposed to be a selective ligand for a muscarinic receptor subtype, was 3-fold higher in competition binding assays carried out with membranes of 1321N1 cells, than with NG108-15 cells. The Hill coefficients of pirenzepine competition curves were not significantly different from unity in both cell lines. This selectivity of pirenzepine was also apparent in studies of the competitive inhibition of carbachol-induced attenuation of cyclic AMP accumulation in intact cells. Differences in the relative affinities of agonists were observed in competition binding analyses carried out with membranes in the presence of GTP and absence of Mg^{2+}. The K_i values of bethanechol and carbachol were 5- and 12-fold lower for receptors of NG108-15 cells than those of 1321N1 cells and the K_i of methacholine was 3.5-fold lower for 1321N1 cells than for NG108-15 cells. The affinities of oxotremorine and arecoline were similar between the two cell lines. These differences in agonist affinities between the two cell lines were much smaller in analyses of muscarinic receptor-mediated effects on cyclic AMP metabolism in intact cells. Taken together, these data suggest that muscarinic receptors of differing pharmacological specificities regulate cyclic AMP metabolism by different mechanisms in 1321N1 and NG108-15 cells.

INTRODUCTION

Binding heterogeneity of muscarinic cholinergic receptor agonists and antagonists has been observed in a variety of preparations (1–7). While complex agonist competition curves with membrane preparations may arise in part from interaction of agonist-occupied receptors with other membrane proteins (5–7), less sanguine statements can be made concerning the origin of heterogeneity of binding observed with the nonclassical muscarinic receptor antagonist pirenzepine. However, based on the tissue selectivity of the antagonism by pirenzepine of muscarinic receptor-mediated physiological responses (4, 8), as well as the reported 30-fold variation in apparent K_i values of this compound determined in radioligand-binding experiments (2), it has been proposed that pirenzepine is a selective antagonist of a putative muscarinic receptor subtype.

The recent availability of radiolabeled pirenzepine has facilitated a direct analysis of the binding of this antagonist in a variety of rat tissues. It has been reported (9, 10) that [^{3}H]PZ[2] binds with high affinity to a subset of muscarinic receptors with a tissue distribution compatible with the concept of distinct M_1 and M_2 receptor subtypes according to the suggested terminology of Goyal and Rattan (11) and Hammer and coworkers (2, 4). That is, [^{3}H]PZ binds with high affinity to a large proportion of the sites (supposedly M_1 receptors) labeled by [^{3}H]

This work was supported by National Institutes of Health Grant GM29536.

[1] Recipient of an Established Investigatorship of the American Heart Association.

[2] The abbreviations used are: PZ, pirenzepine; QNB, (−)-quinuclidinyl benzilate; NMS, *N*-methylscopolamine; DMEM, Dulbecco's modified Eagle's medium; PGE_1, prostaglandin E_1; IBMX, 3-isobutyl-1-methylxanthine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; N_i, the guanine nucleotide binding protein that couples inhibitory receptors to adenylate cyclase.

0026-895X/84/06404-10$02.00/0

QNB in the hippocampus, cortex, and striatum. Little [^{3}H]PZ binding occurs to the muscarinic receptor populations (supposedly M_2 receptors) of heart, cerebellum, and ileum. In tissue preparations where large amounts of [^{3}H]PZ binding are observed, little regulation by guanine nucleotides of agonist binding to muscarinic receptors occurs. Conversely, in preparations that exhibit little [^{3}H]PZ binding, guanine nucleotides markedly reduce the apparent affinity of cholinergic agonists. Based on these results, Watson *et al.* (10) have proposed that [^{3}H]PZ binds more favorably to tissues where little if any effect of guanine nucleotides on agonist binding is observed and have referred to this muscarinic receptor population as the M_1 subtype. Conversely, they proposed that the M_2 receptor subtype represents the muscarinic receptor population that couples to a guanine nucleotide regulatory protein and mediates inhibition of adenylate cyclase.

We recently have initiated a series of experiments designed to define the biochemical properties of muscarinic cholinergic receptors expressed by cultured cell lines. Two cell lines have been identified that exhibit markedly different characteristics regarding the regulation of cyclic AMP metabolism by muscarinic receptors. In intact 1321N1 astrocytoma cells, muscarinic receptor agonists reduce cyclic AMP levels through the calcium-dependent activation of phosphodiesterase (12, 13); no regulation of adenylate cyclase activity by muscarinic receptor agonists is observed in broken cell preparations from these cells (12). In contrast, muscarinic receptor-mediated regulation of cyclic AMP metabolism in NG108-15 neuroblastoma × glioma hybrid cells is mediated by the direct inhibition of adenylate cyclase (14–16). Consistent with these results, pertussis toxin, which ADP-ribosylates the M_r = 41,000 α-subunit of N_i and blocks inhibitory coupling of receptors to adenylate cyclase, has no effect on muscarinic receptor-mediated attenuation of cyclic AMP accumulation in 1321N1 cells, but completely blocks cholinergic effects on cyclic AMP accumulation in NG108-15 cells (17). Since muscarinic receptor subtypes potentially would be coupled to different cellular mechanisms, and since 1321N1 and NG108-15 cells express two biochemical activities proposed to be responsible for muscarinic receptor action, we have compared the pharmacological properties of the muscarinic receptor populations coupled to the cyclic AMP system in each of these cell types. Differences in the selectivity of muscarinic receptor agonists were observed between the two systems; in addition, a small but reproducible difference in the apparent affinity of pirenzepine was detected in membrane preparations from the two cell lines.

MATERIALS AND METHODS

Materials. NG108-15 neuroblastoma × glioma hybrid cells were obtained from Drs. Gary Brooker and Barry Wolfe. DMEM, trypsin, and fetal calf serum were purchased from GIBCO (Grand Island, NY). Tissue culture dishes (150 mm and 12-well cluster plates) were obtained from GIBCO and Falcon. Prostaglandin E_1 was a generous gift from Dr. John Pike, Upjohn (Kalamazoo). Pirenzepine was a gift from Boehringer Ingelheim Ltd. Arecoline, atropine, bethanechol, carbachol, methacholine, oxotremorine, Hepes, Tris, EDTA, (−)-isoproterenol, Dowex 50-X8-400 (H^+ form), and guanosine 5′-triphosphate, sodium salt, were obtained from Sigma Chemical Company (St. Louis, MO). Neutral alumina (Brockman activity 1, 80–200 mesh) was obtained from Fisher Scientific Company (Pittsburgh, PA). [^{3}H]QNB (33.1 Ci/mmol), [^{3}H]NMS (84.8 Ci/mmol) and [^{3}H]PZ (84 Ci/mmol) were from New England Nuclear Corporation (Boston, MA). [^{3}H]Adenine (26–29 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). All other reagents used were of analytical grade.

Cell culture. NG108-15 cells were grown in DMEM supplemented with 10% fetal calf serum, 4.5 g glucose/liter, 0.1 mM hypoxanthine, 16 μM thymidine, and 1 μM aminopterin (16). Cells were grown in a humidified atmosphere of 92% air and 8% CO_2 at 37°. Confluent cultures on 150-mm dishes were used as seeding stocks. Cells were harvested by vigorously pipetting the medium against the dish surface and subcultured at a dilution of approximately 1:10. On the third day after seeding, and each day thereafter, the medium was replaced by fresh medium. Conditions for maintenance of 1321N1 astrocytoma cells were as we previously have described (12, 18).

Membrane preparation. Confluent NG108-15 cell cultures were rinsed once with 50 mM Tris buffer (pH 7.5 at 4°) containing 250 mM sucrose and 5 mM $MgSO_4$ and 5 ml of the same buffer was added to each dish. Cells were detached by scraping with a rubber policeman and then pelleted by centrifugation at 500 × *g* for 5 min. The supernatant was removed and the cell pellet was frozen in liquid N_2 and stored at −80°. A washed membrane fraction was prepared by thawing the cell pellet and homogenizing in 10 ml of 10 mM Hepes buffer containing 10 mM EDTA (pH 7.5 at 4°) with five strokes of a B-type pestle in a Dounce homogenizer. This protocol of freezing, thawing, and homogenization resulted in a greater than 90% cell lysis, as judged by trypan blue exclusion. The homogenate was centrifuged at 500 × *g* for 5 min at 4° to remove nuclei and whole cells. The supernatant was diluted to a final volume of 40 ml with homogenization buffer, and the membranes were centrifuged at 40,000 × *g* for 10 min at 4°. The resulting pellet was resuspended and the wash procedure was repeated. The membrane preparation was stored at −80° until use.

1321N1 astrocytoma cell membranes were prepared by aspirating the growth medium and swelling the cells by the addition of 10 ml of 1 mM Hepes buffer (pH 7.5 at 4°). After 15 min on ice, the cells were scraped from the dish using a rubber policeman and the suspension was centrifuged at 40,000 × *g* for 10 min at 4°. The resulting pellet was resuspended in 10 mM Hepes buffer (pH 7.5 at 4°) containing 10 mM EDTA and homogenized using a Brinkmann Polytron tissue disruptor (setting 6.0 for 15 sec). The homogenate was centrifuged at 40,000 × *g* for 10 min at 4°. This washing procedure was repeated two more times. Membranes not used on the day of preparation were stored at −80° in a small aliquot of 50 mM Tris buffer containing 250 mM sucrose and 5 mM $MgSO_4$ (pH 7.5 at 4°).

Female Sprague-Dawley rats (200–300 g) were killed by decapitation. The heart, cerebral cortex, and cerebellum were dissected and homogenized (approximately 1 g wet weight/20–30 volumes) in 10 mM Hepes buffer containing 10 mM EDTA (pH 7.5 at 4°) using a Brinkmann Polytron tissue disruptor (setting 6.0 for 15 sec). The homogenates were centrifuged at 40,000 × *g* for 10 min at 4° and the pellets were resuspended in the same buffer. This washing procedure was repeated three more times. Membranes were resuspended in 10 mM Hepes buffer containing 5 mM $MgSO_4$ (pH 7.5 at 37°) and used on the day of preparation.

Muscarinic cholinergic receptor assays. Unless specifically stated, all receptor assays were performed in 10 mM Hepes buffer containing 5 mM $MgSO_4$ (pH 7.5 at 37°). Competition binding experiments using [^{3}H]QNB (250–350 pM) or [^{3}H]NMS (200–250 pM) were performed essentially as we have previously described (6, 12) in a final volume of 1 ml in polypropylene tubes (Sarstedt) incubated at 37° for 60 min. Saturation analysis of [^{3}H]QNB (0.5–200 pM) binding was performed in a final volume of 10 ml incubated at 37° for 3–4 hr. Saturation analysis of [^{3}H]NMS (5–250 pM) binding was performed in a final volume of 1 ml incubated at 37° for 2–3 hr. Assays were initiated by

the addition of membranes (1321N1 astrocytoma cells, 500–800 μg protein/assay; NG108-15 cells, 400–600 μg protein/assay; cerebral cortex, 0.4 mg wet weight/assay; cerebellum and heart, 4 mg wet weight/assay) and terminated by the addition of 10 ml of 10 mM Tris buffer (pH 7.5 at 37°) followed by immediate filtration through Schleicher and Schuell No. 30 glass fiber filters. Each filter was washed with a further 10 ml of 10 mM Tris buffer. Nonspecific binding was defined as the amount of radioligand bound in the presence of 1 μM atropine and ranged from 5–10% of the total radioligand retained by the filters. The filters were transferred to glass vials to which 3 ml of Budget Solve (Research Products, Inc.) scintillation fluid was added. The samples were counted in a scintillation counter at 40% efficiency. Under the conditions stated, less than 10% of total radioligand was bound in all experiments. All assays were performed in triplicate.

Analysis of [^{3}H]PZ (0.02–10 nM) binding to cerebral cortical membranes (1 mg wet weight/assay) was carried out in a final volume of 0.25 ml. The assay was initiated by the addition of membranes and continued at 30° for 3 hr. The incubation was terminated by the addition of 10 ml of ice-cold 10 mM Tris buffer (pH 7.5 at 4°) and immediate filtration through Schleicher and Schuell No. 30 glass fiber filters that had been treated with siliconizing agent (Prosil 28) as we have previously described (19). Nonspecific binding was defined as the amount of [^{3}H]PZ bound in the presence of 1 μM atropine and ranged from 15–25% of the total [^{3}H]PZ retained by the filters.

Intact cell binding assays, using [^{3}H]NMS as the radioligand, were carried out using NG108-15 and 1321N1 cells grown in 12-well cluster plates. The growth medium was aspirated and replaced with 0.9 ml of DMEM + 25 mM Hepes (pH 7.5 at 37°). Assays were initiated by the addition of [^{3}H]NMS and terminated by aspiration and two 1-ml washes with ice-cold DMEM + 25 mM Hepes. Radioactivity was solubilized by the addition of 1 ml of 0.5 N NaOH. The samples were neutralized with 1 ml of 0.5 N HCl prior to the addition of 10 ml of scintillation fluid. Samples were counted at 30% efficiency. Incubations were for 90 min at 37° for saturation analysis of [^{3}H]NMS (5–600 pM) binding and for 60 min at 37° in competition binding experiments (200–250 pM). Cell density was such that less than 10% of total added radioligand was bound in all experiments. Nonspecific binding was defined as the amount of radioligand bound in the presence of 1 μM atropine and ranged from 4–8% of total radioactivity bound. All assays were performed in quadruplicate.

There are important differences in binding assays carried out with [^{3}H]NMS and those carried out with [^{3}H]QNB. These differences apparently evolve from the difference in relative lipophilicity of [^{3}H]NMS versus [^{3}H]QNB as previously noted by Galper *et al.* (20). That is, due to its hydrophilicity, [^{3}H]NMS does not readily detect either intravesicular or intracellular receptors. In intact cell binding assays with either 1321N1 cells or NG108-15 cells, [^{3}H]NMS detected approximately the same density of receptors as was detected by [^{3}H]QNB in lysates from these cells. Although a high degree of nonspecific binding was encountered, intact cell binding assays with [^{3}H]QNB also detected approximately the same number of receptors as is detected in intact cell binding assays with [^{3}H]NMS. In contrast, in cell lysates, [^{3}H]NMS detected only 30–50% of the receptors detected by [^{3}H]QNB. Studies on agonist-induced internalization of muscarinic receptors in 1321N1 cells also indicate that [^{3}H]NMS detects only surface receptors, whereas [^{3}H]QNB detects both intracellular and intravesicular receptors.[3] In spite of the difference in the total number of sites labeled with each radioligand in membrane fractions, the calculated affinities obtained for both agonists and antagonists were the same irrespective of the radioligand used. [^{3}H]NMS was utilized in competition binding experiments with agonists in order to circumvent the large shifts of the competition curves to the right (and therefore the very high concentrations of competing drugs) due to the very low K_d of [^{3}H]QNB, i.e., the ratio of radioligand concentration to its K_d is much higher with [^{3}H]QNB than with [^{3}H]NMS.

Measurement of cyclic AMP accumulation in intact cells. A modification (12) of the method of Shimizu *et al.* (21) was used to measure intracellular cyclic AMP levels. NG108-15 and 1321N1 cells were grown in 12-well cluster plates. The growth medium was aspirated and the cells were incubated for 60 min at 37° with 1 ml of DMEM + 25 mM Hepes (pH 7.5 at 37°) containing 1–2 μCi of [^{3}H]adenine per ml. The cells were then washed with two 1-ml aliquots of DMEM/Hepes and a fresh aliquot of 0.95 ml of DMEM/Hepes was added. The cells were allowed to equilibrate for 30 min. Intracellular cyclic AMP accumulation was stimulated by PGE_1 (1–10 μM) or isoproterenol (10 μM) and the capacity of various muscarinic cholinergic receptor agonists to inhibit accumulation of nucleotide was examined. Incubations (5–10 min) were terminated by aspiration of the medium and addition of 1 ml of 5% trichloroacetic acid (w/v) containing 0.5 mM cyclic AMP. Separation of the [^{3}H]cyclic AMP formed from [^{3}H]ATP was accomplished by a modification (12) of the method of Salomon *et al.* (22) using sequential chromatography over Dowex and alumina columns. The [^{3}H]cyclic AMP synthesized during the challenge period is expressed as the percentage conversion of [^{3}H]ATP to [^{3}H]cyclic AMP, i.e., (dpm of [^{3}H]cyclic AMP/dpm of [^{3}H]ATP + dpm of [^{3}H]cyclic AMP) × 100. Column recoveries were assessed by spectrophotometric determination (259 nM) of the cyclic AMP concentration in an aliquot of the [^{3}H]cyclic AMP eluate. Recoveries ranged from 50–75%.

Data analysis. Saturation isotherms were transformed using the method of Scatchard (23) and estimates of K_d and B_{max} obtained using unweighted linear regression analysis of the transformed data. Hill slopes and IC_{50} values were calculated from competition binding experiments and the IC_{50} values were transformed to apparent K_i values using the method of Cheng and Prusoff (24). When pirenzepine competition curves yielded Hill slopes that were significantly different from unity (i.e., in the cerebral cortex) the binding data were analyzed by nonlinear least squares regression analysis using the Gauss-Newton method (25). The model used was that for law of mass action binding of the competing ligand with either a single site ($n = 1$) or two independent sites ($n = 2$) as follows:

$$B_S = \sum_{i=1}^{n} \left[B_i - \frac{B_i S}{IC_{50i} + S} \right] + N$$

in which S is the concentration of competing ligand, B_S is the [^{3}H]QNB bound at a given concentration of S, B_i is the number of specific binding sites, IC_{50i} is the IC_{50} for the competing ligand at each binding site, and N is the number of nonspecific binding sites. Computer-derived IC_{50} values were corrected to apparent K_i values as described above. To determine if the data were fit significantly better by the two-site model, the residual sums of squares of the respective fits were compared using a partial F test (26).

Protein assay. Protein concentration was determined by the method of Lowry *et al.* (27) using bovine serum albumin as a standard.

RESULTS

The interaction of the two radiolabeled muscarinic receptor antagonists, [^{3}H]QNB and [^{3}H]NMS, with receptors of the two cell lines was investigated. Saturation binding data were transformed using the method of Scatchard (23) and representative plots for [^{3}H]QNB are shown in Fig. 1. [^{3}H]QNB bound with high affinity to a single class of binding sites in both cell lines ($K_d = 6.4 \pm 1.2$ pM in NG108-15 cells; $K_d = 6.3 \pm 1.1$ pM in 1321N1 cells; mean ± standard error, $n = 3$). The estimated K_d values are similar to values that we have previously reported for the binding of [^{3}H]QNB to membrane preparations derived from a number of rat brain regions (7) and rat heart (6). Similarly, [^{3}H]NMS bound with the same high affinity (data not shown) to a single class of binding sites in NG108-15 and 1321N1 cell membrane preparations ($K_d = 49 \pm 17$ pM in NG108-15 cells; $K_d =$

[3] T. K. Harden, unpublished.

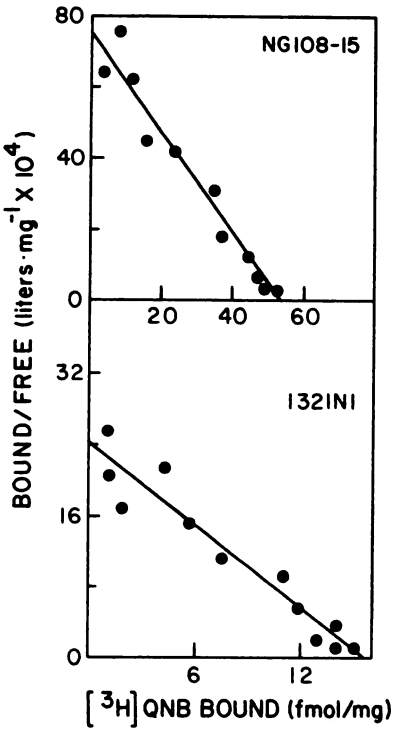

FIG. 1. *Scatchard transforms of [^{3}H]QNB binding to NG108-15 and 1321N1 cell membranes*

Membrane preparation and saturation analysis of [^{3}H]QNB (0.5–200 pM) binding were performed as described in Materials and Methods. Data are representative of three similar experiments for each cell line.

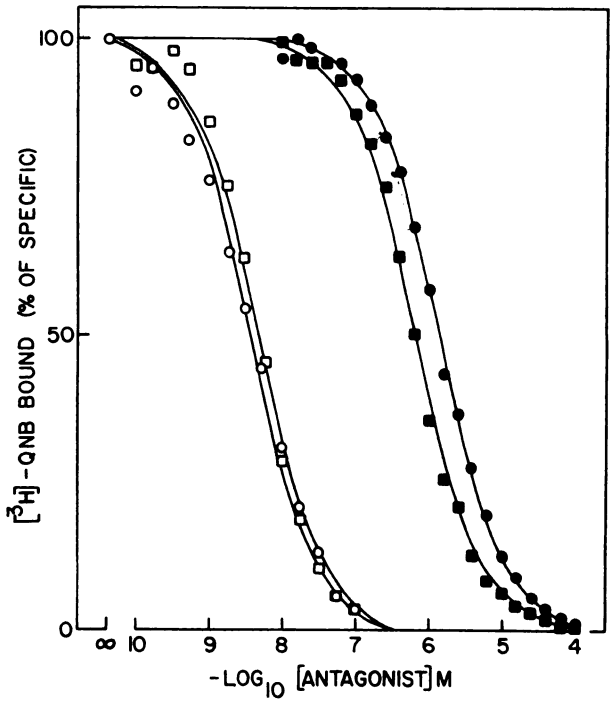

FIG. 2. *Inhibition of [^{3}H]QNB binding to membranes of NG108-15 and 1321N1 cells by atropine and pirenzepine*

Membranes were prepared as described in Materials and Methods. [^{3}H]QNB (300–350 pM) binding to membranes of 1321N1 (□, ■) and NG108-15 cells (○, ●) was determined in the presence of the indicated concentrations of atropine (open symbols) and pirenzepine (closed symbols). IC_{50} values were converted to apparent K_i values using the Cheng and Prusoff (24) correction. These values and the calculated Hill coefficients for the pirenzepine competition curves are shown in Table 1. The curves drawn represent the computer-derived fits of the data to a single site model. The inclusion of a second site did not significantly improve the fit of the data. Mean and range pirenzepine K_i values were 10.7 nM (10–11.9, $n = 6$) and 25 nM (23–29.6, $n = 7$) for 1321N1 and NG108-15 cells, respectively. Mean and range atropine K_i values were 0.08 nM (0.06–0.1, $n = 6$) and 0.08 nM (0.06–0.09, $n = 7$) for 1321N1 and NG108-15 cells, respectively.

53 ± 11 pM in 1321N1 cells; $n = 3$). [^{3}H]NMS bound to only 30–50% of the sites labeled by [^{3}H]QNB in membrane preparations. As is discussed in Materials and Methods, this result is apparently due to the relative hydrophilicity (20) of [^{3}H]NMS and the sequestration of receptors inside vesicles in the membrane preparation.

Since pirenzepine has been proposed to interact with putative muscarinic receptor subtypes with different affinities, the binding properties of this antagonist were determined in the two cell lines and compared to its binding properties in membrane preparations from rat cerebral cortex, cerebellum, and heart. The competition binding data obtained with pirenzepine also was compared to that obtained with the classical muscarinic receptor antagonist, atropine. Representative competition curves for the two antagonists binding to membranes of 1321N1 and NG108-15 cells are shown in Fig. 2 and the apparent K_i values and Hill coefficients are presented in Table 1. As has been previously reported (2), pirenzepine competed with [^{3}H]QNB in a complex fashion (i.e., Hill coefficients were significantly less than unity) in membrane preparations from the cerebral cortex. Unweighted nonlinear least squares regression analysis of the competition curves demonstrated that the data were significantly better fit by a two-, rather than a single-site model with a high affinity ($K_H = 8.0 \pm 2.1$ nM; 75 ± 7% of total sites; $n = 5$) and a low affinity ($K_L = 93 \pm 43$ nM; 25 ± 7% of total sites; $n = 5$) binding component. In contrast to the cerebral cortex, pirenzepine competition curves with membrane preparations from the cerebellum and heart exhibited Hill coefficients that were not significantly different from unity. The apparent K_i values, 93 nM in cerebellum and 147 nM in heart, were much greater than the high affinity binding component observed in cerebral cortex, and demonstrate the marked tissue selectivity of the inhibition of radioligand binding by pirenzepine. Atropine competed with [^{3}H]QNB with K_i values of 0.18 nM in the cerebral cortex and 0.46 nM in the heart and in a manner compatible with law of mass action kinetics of a ligand interaction with a single receptor affinity state in both tissues (data not shown). The slightly higher affinity of atropine observed in cerebral cortical membranes is consistent with the report of Hammer and Giachetti (4) who observed a 2.9-fold difference in the apparent K_i for atropine in competition binding experiments with [^{3}H]NMS in rat sympathetic ganglia and atria.

In light of the fact that muscarinic receptors of NG108-15 cells couple to adenylate cyclase while those of 1321N1 cells do not, it was anticipated that pirenzepine might compete for [^{3}H]QNB binding with high affinity (like that in the cerebral cortex) at muscarinic receptors of 1321N1 astrocytoma cells and with low affinity (like that

TABLE 1

K_i values and Hill coefficients derived from the inhibition by pirenzepine of [^{3}H]QNB binding to membranes of NG108-15 and 1321N1 cells and membranes of rat cerebral cortex, cerebellum, and heart

Membrane preparation and competition binding experiments, using [^{3}H]QNB as a radioligand, were performed as described in Materials and Methods. K_i values were calculated using the Cheng and Prusoff (24) correction of estimated IC_{50} values. "K_i" represents corrected IC_{50} values for the unresolved pirenzepine competition curves in cerebral cortical membranes. Each vertical bar represents an individual experiment. When pirenzepine competition curves yielded Hill slopes that were significantly different from unity (i.e., cerebral cortex), the binding data were analyzed as described in Materials and Methods. K_H and K_L refer to the K_i values of the high and low affinity binding components of five detailed pirenzepine/[^{3}H]QNB competition curves in cortex. Hill slopes (nH, mean ± standard error) were calculated from the competition curves by plotting log (fraction bound/1 − fraction bound) versus log (pirenzepine).

TISSUE	−LOG [PIRENZEPINE]	MEAN Ki nM	nH
K_H CORTEX		8	
"Ki" CORTEX		13	0.85±0.02
K_L CORTEX		93	
Ki 1321N1		11	1.04±0.03
Ki NG108-15		25	1.02±0.05
Ki CEREBELLUM		93	1.06±0.04
Ki HEART		147	1.14±0.04
	8 7		

observed in the heart) at the muscarinic receptors of NG108-15 cells. Pirenzepine did bind with high affinity (K_i = 11 nM) to receptors of 1321N1 cells (Fig. 2, Table 1). Furthermore, binding was apparently to a homogeneous class of binding sites in membranes from 1321N1 cells (Table 1). The Hill coefficients of pirenzepine competition curves were approximately 1 and computer analysis of detailed curves (Fig. 2) indicated that these curves were not significantly better fit to the equation for two binding sites. The antagonist also bound with relatively high affinity (25 nM) to muscarinic receptors of NG108-15 cells (Fig. 2). Thus, only a small, but reproducible, difference in the apparent K_i values between the two cell lines was observed.[4] As with 1321N1 cells, pirenzepine apparently interacts with a homogeneous class of binding sites in NG108-15 cells as indicated by Hill slopes of approximately 1 (Table 1) and computer analysis of detailed pirenzepine competition curves (Fig. 2). Atropine bound with the same high affinity to a single class of binding sites in both cell lines.

The competitive inhibition by pirenzepine of carbachol-induced decreases in agonist-stimulated cyclic AMP accumulation was investigated in intact cells (Fig. 3). PGE_1 and isoproterenol were used to evaluate cyclic AMP levels in NG108-15 and 1321N1 cells, respectively. The apparent K_i values for the antagonism by pirenzepine of the muscarinic receptor-mediated inhibition of cyclic AMP accumulation were calculated using the Cheng and Prusoff (24) correction of the estimated IC_{50} values. The results are qualitatively in excellent agree-

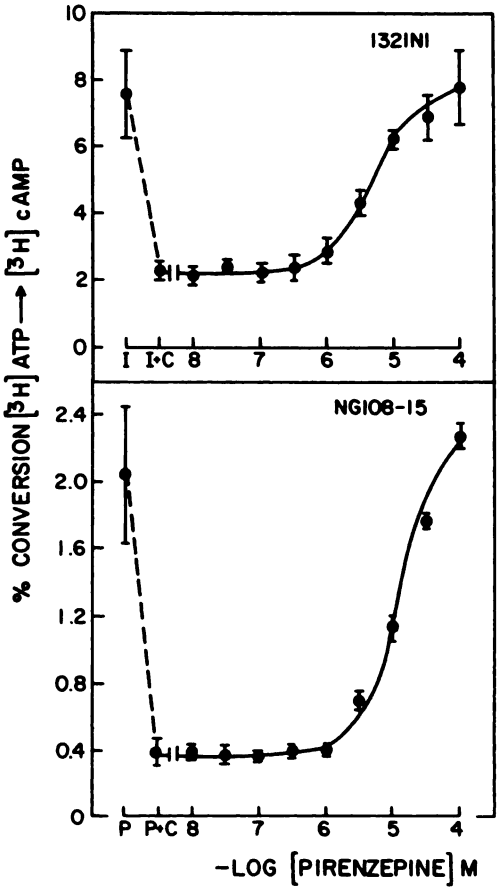

FIG. 3. *Competitive inhibition by pirenzepine of carbachol-induced attenuation of cyclic AMP accumulation in intact 1321N1 and NG108-15 cells*

Cell culture and measurement of intact cell cyclic AMP accumulation were performed as described in Materials and Methods. Cyclic AMP synthesis was stimulated by 10 μM isoproterenol (*I*; 1321N1 cells) or 1 μM PGE_1 (*P*; NG108-15 cells) and stimulated levels were inhibited by 100 μM carbachol (*C*). The carbachol-induced attenuation was competitively reversed by increasing concentrations of pirenzepine. The apparent K_i values for pirenzepine were calculated by the Cheng and Prusoff (24) correction of the estimated IC_{50} values (i.e., that concentration of pirenzepine that effectively reversed 50% of the inhibition produced by 100 μM carbachol) using the respective $K_{0.5}$ values for carbachol in the two cell lines as shown in Table 3. In 1321N1 cells, the mean K_i value was 35 nM (range 19–58, n = 4) and in NG108-15 cells the mean K_i value was 96 nM (range 74–130 nM, n = 6).

[4] A logical extension of these indirect studies of the binding of pirenzepine was to directly assess the binding of [^{3}H]PZ to the muscarinic receptor populations of 1321N1 and NG108-15 cells. To this end, a binding assay for [^{3}H]PZ was established (as described in Materials and Methods) using membrane preparations derived from the rat cerebral cortex. In good agreement with observations from other laboratories (9, 10, 34), [^{3}H]PZ bound with high affinity (K_d = 2.14 ± 0.61 nM, n = 8) to a subset (approximately 50–60% of the sites labeled with [^{3}H]QNB) of the muscarinic receptors. The direct binding of [^{3}H]PZ to membranes from the two cell lines proved to be technically difficult due to high nonspecific binding (relative to that observed in cerebral cortex) in such preparations. However, in two experiments using 1321N1 astrocytoma cell membranes, [^{3}H]PZ labeled a very high (>90%) proportion of the sites labeled by [^{3}H]QNB. Conversely, in these experiments using NG108-15 cell membranes [^{3}H]PZ labeled a very small percentage, if any, of the sites labeled by [^{3}H]QNB.

ment with the relative K_i values derived from the competition binding studies (Fig. 2, Table 1). That is, pirenzepine antagonized muscarinic receptor stimulation with higher affinity in the 1321N1 cell line (K_i = 35 nM in 1321N1 cells; K_i = 96 nM in NG108-15 cells). Thus, the values determined by intact cell analysis are of lower affinity than the values obtained in radioligand-binding studies (Table 1), but the fold-selectivity of pirenzepine between the two systems was similar.

Finally, pirenzepine affinity in the two cell lines was examined in intact cell binding assays using [^{3}H]NMS (Table 2). The K_i obtained for pirenzepine in intact cell assays with NG108-15 cells (Table 2) was in very good agreement with the K_i obtained by antagonism of cholinergic agonist-mediated attenuation of cyclic AMP accumulation in these cells (Figure 3). In contrast, the K_i determined in intact cell binding assays with 1321N1 cells was approximately 3-fold greater than the K_i determined in the same cells measuring blockade of cholinergic effects on cyclic AMP accumulation. Thus, little selectivity of pirenzepine between the two cell lines was detected in intact cell binding assays. In contrast to the result obtained in membrane binding assays, atropine exhibited an approximately 2-fold higher affinity in intact cell binding assays with NG108-15 cells than with 1321N1 cells.

The affinities of five agonists for the muscarinic cholinergic receptors expressed by the two cell lines were determined in competition binding experiments using [^{3}H]NMS (Fig. 4, Table 3). To minimize the formation of a high affinity, guanine nucleotide-sensitive, agonist-receptor-guanine nucleotide regulatory protein complex, these experiments were performed in the absence of any added Mg^{2+} and in the presence of 500 μM GTP. Under such conditions, the Hill coefficients of competition curves for all agonists in both cell lines were not significantly different from unity, indicating the interaction of the ligands with a single affinity state of the receptor. In NG108-15 cell membranes, the rank order of potency for agonist affinity was oxotremorine > arecoline ≥ carbachol > bethanechol > methacholine (Table 3). In paired experiments, the potency order of the same agonists in 1321N1 cell membranes was markedly different with oxotremorine > arecoline > methacholine > carbachol > bethanechol. Thus, bethanechol and carbachol bound with higher affinity to receptors of NG108-15 cell membranes (approximately 5- and 12-fold, respectively) while methacholine bound with higher (3.5-fold) affinity to receptors of 1321N1 cell membranes. Arecoline and oxotremorine were only slightly selective, binding with higher affinity to receptors of NG108-15 cells (2.2- and 1.5-fold, respectively). The affinities of carbachol and oxotremorine determined using [^{3}H]QNB as the radioligand (data not shown) were essentially identical to the values for these agonists determined using [^{3}H]NMS (Table 3).

The capacity of these muscarinic receptor agonists to attenuate agonist-stimulated cyclic AMP accumulation also was examined in the two cell lines. The results of these experiments are shown in Table 3 where the data are expressed as $K_{0.5}$ values, i.e., that concentration of muscarinic receptor agonist that produced 50% of the inhibition elicited by a maximally effective agonist concentration. All five agonists inhibited cyclic AMP accumulation to approximately the same extent at maximally effective concentrations, i.e., all were "full" agonists. In contrast to the K_i values determined in membrane binding assays, bethanechol, carbachol, and methacholine exhibited little, if any, selectivity in their ability to inhibit cyclic AMP accumulation in the two cell systems. Arecoline and oxotremorine were approximately 2–3-fold more potent in NG108-15 cells.

TABLE 2

[^{3}H]NMS binding to intact 1321N1 and NG108-15 cells

Saturation and competition binding experiments were carried out as described in Materials and Methods. K_d values represent mean ± standard error. K_i values were calculated using the Cheng and Prusoff (24) correction of the estimated IC_{50} values. Values represent means of the indicated number (n) of experiments. The range of experimental values is also presented.

	1321N1	NG108-15
[^{3}H]NMS K_d (pM)	53 ± 6 (n = 4)	41 ± 4 (n = 3)
Atropine K_i (nM)	0.61 (0.53–0.72, n = 5)	0.30 (0.24–0.35, n = 4)
Pirenzepine K_i (nM)	103 (75–159, n = 4)	137 (98–167, n = 4)

DISCUSSION

Although extensive information concerning muscarinic cholinergic receptors has accumulated through the use of radioligand binding assays in recent years, crucial information regarding this receptor is still not available. Foremost in this deficit in our knowledge is the meaning of the different affinity states of the muscarinic receptor encountered with a variety of ligands. Complex binding curves for ligand interaction at these receptors most often have been explained either by the existence of muscarinic receptor subtypes or by the existence of conformational states arising from the coupling of a single population of muscarinic receptors to membrane effector proteins. While unambiguous evidence for muscarinic receptor subtypes has not been reported, the contribution of guanine nucleotide regulatory protein(s) accounts at least in part for the heterogeneous binding properties of cholinergic agonists.

Many of the uncertainties regarding complex binding of ligands to muscarinic receptors have evolved from the use of membranes prepared from tissues of heterogeneous cellular content. The use of cultured cell lines in the current work circumvents this concern. An equally important impetus for the present work evolved from the properties of the muscarinic receptor populations expressed by the two cell lines. A variety of evidence indicates that activation of muscarinic cholinergic receptors of 1321N1 cells reduces intracellular cyclic AMP levels through activation of phosphodiesterase (12, 13). No evidence for inhibition of adenylate cyclase by muscarinic receptor agonists has been observed in 1321N1 cells, and pertussis toxin, which blocks inhibitory coupling of receptors to adenylate cyclase, has no effect on

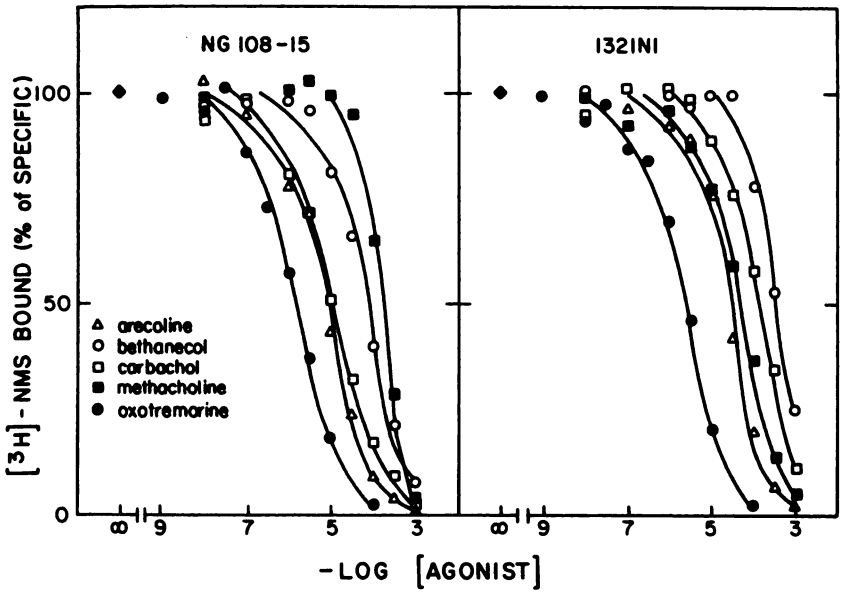

FIG. 4. *Inhibition of [³H]NMS binding to 1321N1 and NG108-15 cell membranes by muscarinic receptor agonists*

Membranes were prepared as described in Materials and Methods except that the final resuspension was in 10 mM Hepes (pH 7.5 at 37°) with no added Mg^{2+}. Competition binding experiments, using [³H]NMS (200–240 pM) as the radioligand, were performed as described in the text except that all assays contained 500 μM GTP. Apparent K_i values (shown in Table 3) were calculated using the Cheng and Prusoff (24) correction of estimated IC_{50} values.

TABLE 3

Apparent affinity constants for the interaction of agonists with muscarinic receptors of intact cell and membrane preparations of NG108-15 and 1321N1 cells

Membranes were prepared as described in Materials and Methods except that the final resuspension was with 10 mM Hepes (pH 7.5 at 37°) with no added Mg^{2+}. Competition binding assays were performed as described in the text except that all assays contained 500 μM GTP. The agonist affinities for the two cell lines are presented as apparent K_i values and were calculated using the Cheng and Prusoff (24) correction of the estimated IC_{50} values. Measurement of intact cell cyclic AMP accumulation was performed as described in the text. Nucleotide accumulation was stimulated by 1 μM PGE_1 (NG108-15 cells) or 10 μM ISO (1321N1 cells) and inhibited by increasing concentrations of the muscarinic agonists. Data are presented as $K_{0.5}$ values (i.e., that concentration of muscarinic agonist that elicits 50% of the inhibition produced by a maximally effective agonist concentration). Values (K_i and $K_{0.5}$) represent the means of the respective n values. The range of experimental values are also presented.

Preparation and cell line	Muscarinic receptor agonist				
	Arecoline	Bethanechol	Carbachol	Methacholine	Oxotremorine
Membranes					
NG108-15					
K_i (μM)	2.2	10.5	2.4	29.6	0.40
Range	(1.6–2.9)	(8.5–13.2)	(1.7–4.1)	(24.0–33.9)	(0.2–0.7)
n	(5)	(4)	(5)	(3)	(5)
1321N1					
K_i (μM)	4.9	55.9	28.1	8.6	0.6
Range	(3.6–6.9)	(45.6–69.5)	(18.2–35.2)	(7.2–9.8)	(0.4–0.9)
n	(5)	(4)	(5)	(4)	(5)
1321N1/NG108-15	2.2	5.3	11.7	0.3	1.5
Intact cells					
NG108-15					
$K_{0.5}$ (μM)	0.6	24.9	0.7	0.4	0.08
Range	(0.3–1.3)	(9.0–63.1)	(0.4–1.0)	(0.2–0.6)	(0.05–0.1)
n	(6)	(3)	(4)	(3)	(6)
1321N1					
$K_{0.5}$ (μM)	1.7	27.5	0.84	0.3	0.3
Range	(1.6–1.8)	(23.4–30.2)	(0.5–2.9)	(0.2–0.4)	(0.1–0.8)
n	(3)	(3)	(5)	(5)	(6)
1321N1/NG108-15	2.8	1.1	1.2	1.2	3.5

muscarinic receptor-mediated attenuation of cyclic AMP accumulation in 1321N1 cells (17). One potential conclusion from these findings is that the muscarinic receptor population of 1321N1 cells is not capable of coupling to the components of the adenylate cyclase system. In this regard, we have recently observed marked effects of muscarinic receptor stimulation on phosphoinositide breakdown and $^{45}Ca^{2+}$ efflux in 1321N1 cells (28). The working hypothesis that has evolved from these studies is that the muscarinic receptor population of 1321N1 cells couples to the phosphoinositide/Ca^{2+} system and does not interact with adenylate cyclase.

Further evidence for lack of interaction of muscarinic receptors of 1321N1 cells with components of the adenylate cyclase has recently been obtained. Guanine nucleotide-sensitive high affinity agonist binding to these receptors has been observed, and the capacity of agonists to form this binding state is closely correlated with their efficacies for stimulation of phosphoinositide breakdown and $^{45}Ca^{2+}$ efflux.[5] Unlike the muscarinic receptors of NG108-15 cells and other receptors that have been shown to negatively couple to adenylate cyclase, the GTP-sensitive agonist binding to muscarinic receptors in 1321N1 cells is unaffected by concentrations of pertussis toxin that maximally ADP-ribosylate and inactivate N_i.[5] Thus, although functional N_i is present in 1321N1 cells (17), the muscarinic receptors of these cells apparently interact with a different guanine nucleotide regulatory protein. This interaction may be important for cholinergic stimulation of phosphoinositide breakdown.

We (16) and others (14, 15) have shown that muscarinic receptor stimulation in NG108-15 cells results in inhibition of adenylate cyclase. This interaction is reflected by marked effects of guanine nucleotides on agonist binding to muscarinic receptors (16). In contrast to 1321N1 cells, treatment of NG108-15 cells with pertussis toxin completely blocks the inhibitory activity of cholinergic agonists (17). Whether this is the only activity of muscarinic receptors or whether this is the only population of muscarinic receptors of NG108-15 cells is brought into question by the preliminary data of Siman and Klein (29) who have reported that a muscarinic receptor-stimulated increase in phosphoinositide turnover occurs in these cells. Furthermore, the recent report of Akiyama *et al.* (30) adds uncertainty to the status of the muscarinic receptor population(s) of NG108-15 cells. These workers have reported that pirenzepine competition curves can be modeled to a two-site binding model with 72% of the interaction occurring at a site with high affinity similar to that observed in cerebral cortex. It is unclear why this heterogeneity was not observed in the current study. Nonetheless, the work of Akiyama *et al.* (30) as well as the possibility that a phosphoinositide response to cholinergic stimuli occurs in these cells establishes the possibility that more than a single receptor subtype exists on these cells. If so, it must be argued that in our hands pirenzepine does not bind with differing affinities to these putative subtypes on NG108-15 cells. Furthermore, the determination of a binding constant for pirenzepine in studies of cyclic AMP accumulation in NG108-15 cells would circumvent such heterogeneity since this response should be mediated by a single receptor type.

The source of binding heterogeneity of pirenzepine in previous work has been proposed to be due to the differential affinity of this nonclassical antagonist for muscarinic receptor subtypes (2, 4, 10). Indeed, a terminology for putative muscarinic receptor subtypes ("M_1" for receptors that bind pirenzepine with high affinity and "M_2" for receptors that bind pirenzepine with low affinity) is in part based on this work. Two points need to be made concerning the present data in relation to that obtained in previous studies with rat tissues. First, pirenzepine competition curves with each cell line followed law of mass action kinetics for interaction at a single binding site. Since the heterogeneous binding of pirenzepine in previous studies with rat tissues has been proposed to result from different affinities of the drug for various proportions of receptor subtypes in the tissues studied, these data can be construed to mean that a single muscarinic receptor population exists on each of the cell lines. The second point of concern is the relative affinities of the drug for the muscarinic receptors of each of the cell lines. In six paired experiments, the affinity of pirenzepine for muscarinic receptors of 1321N1 cells was always approximately 3-fold higher than for the receptors of NG108-15 cells. Since these assays were carried out under essentially identical conditions in regard to tissue and receptor concentration and since the affinity of atropine in the same experiments (and [^{3}H]QNB and [^{3}H]NMS in other experiments) was not different between the receptors of the two cell lines, this difference in affinity of pirenzepine between the two cell lines is not trivial. This affinity difference was also supported by assays of cyclic AMP accumulation in intact cells (Fig. 3). Although pirenzepine affinities determined in intact cell binding assays did not greatly differ betwen the two cell lines, the affinity of atropine differed by 2-fold. Thus, relative to atropine (which did not differ in affinity in membrane assays with the two cell lines), about the same degree of "selectivity" of pirenzepine was observed between the two cell lines in intact cell binding assays.

One interpretation of these pharmacological data is that pirenzepine interacts with one affinity with a muscarinic receptor subtype on 1321N1 cells and a lower affinity with another muscarinic receptor subtype on NG108-15 cells. However, uncertainties remain in this interpretation. Hammer and coworkers (2, 4) have discussed differences in affinity of pirenzepine for putative receptor subtypes of 10–30-fold; at best only a 3-fold difference was observed with the two cell lines. In support of our results, Gil and Wolfe (31) have reported in a preliminary communication that atropine and scopolamine exhibited a 4–6-fold selectivity for blockade of muscarinic receptor-mediated effects on phosphoinositide breakdown versus adenylate cyclase activity whereas pirenzepine exhibited a 16-fold selectivity for the phosphoinositide response. Thus, in their hands, the "net selectivity" of pirenzepine between the two systems was approximately 3-fold. Hammer and Giachetti (4) also

[5] T. Evans, J. R. Hepler, S. L. Brown, J. H. Brown, and T. K. Harden, unpublished observations.

have observed a 3-fold selectivity for atropine between tissues that they proposed expressed M_1-receptors and those that expressed M_2-receptors. Thus, the "fold-selectivity" of pirenzepine observed relative to atropine and other "classical" antagonists may be somewhat less than the 10–30-fold selectivity claimed in some studies. In regard to absolute affinity, the interaction of pirenzepine with muscarinic receptors of 1321N1 cells occurred with an affinity similar to the high affinity component of the binding of this drug to cerebral cortical receptors (Fig. 2; Table 1). Taken together, these data are consistent with the idea, but certainly do not prove the existence, of separate muscarinic receptor subtypes on 1321N1 and NG108-15 cells. Alternatively, more than two muscarinic receptor subtypes may exist (32), or as has been recently proposed by Roeske and Venter (33) pirenzepine may simply bind to an allosteric state of the muscarinic receptor.

The paucity of information concerning the relative affinities of agonists for binding to muscarinic receptors in tissues other than those that exhibit considerable cellular heterogeneity led us to undertake a comparative study of agonists with the two cell lines. Hill coefficients of approximately unity for the agonist competition curves indicate that predominantly a single low affinity "noncoupled" state of muscarinic receptors was observed in these experiments. To our knowledge, the agonist-binding data (Table 3) represents the first comparison of agonist-binding properties between tissues whose muscarinic receptor interactions apparently occur by law of mass action kinetics to a single population of sites. The K_i values of three agonists (bethanechol, carbachol, and methacholine) were markedly different in comparative binding assays with membranes from the two cell lines (Table 3). This apparent difference in affinities was not as obvious in analyses of cyclic AMP accumulation in intact cells. However, such a result is not surprising in light of the fact that multicomponent systems are involved in the regulation of cyclic AMP levels by muscarinic receptors in each cell line. Furthermore, in 1321N1 cells, oxotremorine and arecoline are much less efficacious agonists than carbachol and methacholine in regard to stimulation of phosphoinositide turnover and $^{45}Ca^{2+}$ efflux (28). If these phenomena are reflective of earlier steps in the series of reactions resulting in activation of phosphodiesterase, a considerable degree of "receptor spareness" may be operative in this system. The fact that the $K_{0.5}$ values for carbachol and methacholine inhibition of cyclic AMP accumulation are approximately 30-fold less than their K_i values for binding, while these two values are similar for oxotremorine and arecoline, is consistent with the premise of receptor spareness and the difference in efficacy of these four agonists in regard to effects on phosphoinositide turnover and $^{45}Ca^{2+}$ mobilization. Thus, while marked differences in agonist affinities were not observed in the intact cell analyses, there is little reason to believe that the differences observed with the same agonists in competition binding assays do not reflect real differences in the properties of the receptors of the two cell lines.

In summary, substantial arguments can be made for the usefulness of 1321N1 and NG108-15 cells as model systems for the study of muscarinic receptors. The muscarinic receptors of these two cell lines are apparently coupled to different biochemical mechanisms. The nonclassical antagonist pirenzepine, as well as several agonists, exhibit some selectivity between the receptor populations of the two cells. One interpretation of these results is that muscarinic receptor subtypes exhibiting different pharmacological specificities are present on these cells.

ACKNOWLEDGMENTS

The authors are grateful to Angie Hodgin for excellent assistance in preparing the manuscript and Gwen Boyd for maintenance of the cell cultures.

REFERENCES

1. Birdsall, N. J. M., A. S. V. Burgen, and E. C. Hulme. The binding of agonists to brain muscarinic receptors. *Mol. Pharmacol.* **14**:723–726 (1978).
2. Hammer, R., C. P. Berrie, N. J. M. Birdsall, A. S. V. Burgen, and E. C. Hulme. Pirenzepine distinguishes between different subclasses of muscarinic receptors. *Nature* **283**:90–92 (1980).
3. Ehlert, R. J., W. R. Roeske, and H. I. Yamamura. Muscarinic cholinergic receptor heterogeneity. *Trends Neurosci.* **5**:336–339 (1982).
4. Hammer, R., and A. Giachetti. Muscarinic receptor subtypes: M_1 and M_2. Biochemical and functional characterization. *Life Sci.* **31**:2991–2998 (1982).
5. Birdsall, N. J. M., C. P. Berrie, A. S. V. Burgen, and E. C. Hulme. Modulation of the binding properties of muscarinic receptors: evidence for receptor-effector coupling, in *Receptors for Neurotransmitters and Peptide Hormones* (G. Pepeu, M. J. Kuhar and S. J. Enna, eds.). Raven Press, New York, 107–116 (1980).
6. Harden, T. K., A. G. Scheer, and M. M. Smith. Differential modification of the interaction of cardiac muscarinic cholinergic and *beta*-adrenergic receptors with a guanine nucleotide binding component(s). *Mol. Pharmacol.* **21**:570–580 (1982).
7. Korn, S. J., M. W. Martin, and T. K. Harden. *N*-Ethylmaleimide-induced alteration in the interaction of agonists with muscarinic cholinergic receptors of rat brain. *J. Pharmacol. Exp. Ther.* **224**:118–126 (1983).
8. Hirschowitz, B. I., J. Fong, and E. Molina. Effects of pirenzepine and atropine on vagal and cholinergic gastric secretion and gastrin release and on heart rate in the dog. *J. Pharmacol. Exp. Ther.* **225**:263–268 (1983).
9. Watson, M., W. R. Roeske, and H. I. Yamamura. [^{3}H]Pirenzepine selectively identifies a high affinity population of muscarinic cholinergic receptors in the rat cerebral cortex. *Life Sci.* **31**:2019–2023 (1982).
10. Watson, M., H. I. Yamamura, and W. R. Roeske. A unique regulatory profile and regional distribution of [^{3}H]-pirenzepine binding in the rat provide evidence for distinct M_1 and M_2 muscarinic receptor subtypes. *Life Sci.* **32**:3001–3011 (1983).
11. Goyal, R. K., and S. Rattan. Neurohumoral, hormonal, and drug receptors for the lower esophageal sphincter. *Gastroenterology* **74**:598–619 (1978).
12. Meeker, R. B., and T. K. Harden. Muscarinic cholinergic receptor-mediated activation of phosphodiesterase. *Mol. Pharmacol.* **22**:310–319 (1982).
13. Meeker, R. B., and T. K. Harden. Muscarinic cholinergic receptor-mediated control of cyclic AMP metabolism. Agonist-induced changes in nucleotide synthesis and degradation. *Mol. Pharmacol.* **23**:384–392 (1983).
14. Hamprecht, B. Structural, electrophysiological, biochemical, and pharmacological properties of neuroblastoma-glioma cell hybrids in cell culture. *Int. Rev. Cytol.* **49**:99–170 (1977).
15. Kurose, H., T. Katada, T. Amano, and M. Ui. Specific uncoupling by islet-activating protein, pertussis toxin, of negative signal transduction via α-adrenergic, cholinergic, and opiate receptors in neuroblastoma × glioma hybrid cells. *J. Biol. Chem.* **258**:4870–4875 (1983).
16. Smith, M. M., and T. K. Harden. Modification of receptor-mediated inhibition of adenylate cyclase in NG108-15 neuroblastoma × glioma cells by *N*-ethylmaleimide. *J. Pharmacol. Exp. Ther.* **228**:425–433 (1984).
17. Hughes, A. R., M. W. Martin, and T. K. Harden. Pertussis toxin differentiates between two mechanisms of attenuation of cyclic AMP accumulation by muscarinic cholinergic receptors. *Proc. Natl. Acad. Sci. U.S.A.*, **81**:5680–5684 (1984).
18. Harden, T. K., S. J. Foster, and J. P. Perkins. Differential expression of components of the adenylate cyclase system during growth of astrocytoma cells in culture. *J. Biol. Chem.* **254**:4416–4422 (1979).
19. Harden, T. K., R. B. Meeker, and M. W. Martin. Interaction of a radiolabelled agonist with cardiac muscarinic cholinergic receptors. *J. Pharmacol. Exp. Ther.* **227**:570–577 (1983).
20. Galper, J. B., L. C. Dziekan, D. S. O'Hara, and T. W. Smith. The biphasic response of muscarinic cholinergic receptors in cultured heart cells to agonists: effects on receptor number and affinity in intact cells and homogenates. *J. Biol. Chem.* **247**:10344–10356 (1982).

21. Shimizu, H., J. W. Daly, and C. R. Creveling. A radioisotopic method for measuring the formation of adenosine 3′,5′-cyclic monophosphate in incubated slices of brain. *J. Neurochem.* **16**:1609–1619 (1969).
22. Salomon, Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548 (1974).
23. Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51**:660–672 (1949).
24. Cheng, Y.-C., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
25. SAS Institute, Inc. *SAS Users Guide: Statistics.* SAS Institute, Cary, NC, 15–39 (1982).
26. DeLean, A., A. A. Hancock, and R. J. Lefkowitz. Validation and statistical analysis of a computer modeling method for quantitative analysis of radioligand binding data for mixtures of pharmacological receptor subtypes. *Mol. Pharmacol.* **21**:5–16 (1982).
27. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
28. Masters, S. B., T. K. Harden, and J. H. Brown. Relationships between phosphoinositide and calcium responses to muscarinic agonists in astrocytoma cells. *Mol. Pharmacol.* **25**:149–155 (1984).
29. Siman, R. G., and W. L. Klein. Specificity of muscarinic acetylcholine receptor regulation by receptor activity. *J. Neurochem.* **37**:1099–1108 (1981).
30. Akiyama, K., M. Watson, W. R. Roeske, and H. I. Yamamura. High affinity [^{3}H]pirenzepine binding to putative M_1 muscarinic sites in the neuroblastoma × glioma hybrid cell line (NG108-15). *Biochem. Biophys. Res. Commun.* **119**:289–297 (1984).
31. Gil, D. W., and B. B. Wolfe. Different pharmacological profiles for two muscarinic-mediated responses. *Soc. Neurosci. Abstr.* **9**:581 (1983).
32. Birdsall, N. J. M., and E. C. Hulme. Muscarinic receptor subclasses. *Trends Pharmacol. Sci.* **4**:459–463 (1983).
33. Roeske, W. R., and J. C. Venter. The differential loss of [^{3}H]pirenzepine *vs* [^{3}H](−)quinuclidinylbenzilate binding to soluble rat brain muscarinic receptors indicates that pirenzepine binds to an allosteric state of the muscarinic receptor. *Biochem. Biophys. Res. Commun.* **118**:950–957 (1984).
34. Luthin, G. R., and B. B. Wolfe. Comparison of ^{3}H-pirenzepine and ^{3}H-quinuclidinylbenzilate binding to muscarinic cholinergic receptors in rat brain. *J. Pharmacol. Exp. Ther.* **228**:648–655 (1984).

Send reprint requests to: Dr. T. Kendall Harden, Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, NC 27514.