MOLECULAR PHARMACOLOGY, 26:156–163

Muscarinic Responses and Binding in a Murine Neuroblastoma Clone (N1E-115)

Selective Loss with Subculturing of the Low-Affinity Agonist Site Mediating Cyclic GMP Formation

MICHAEL MCKINNEY, SCOTT STENSTROM, AND ELLIOTT RICHELSON

Departments of Psychiatry and Pharmacology, Mayo Foundation, Rochester, Minnesota 55905

Received February 29, 1984; Accepted June 14, 1984

SUMMARY

Cells of the murine neuroblastoma clone N1E-115 possess muscarinic receptors that influence the intracellular level of cyclic nucleotides. The stimulation of [^{3}H]cyclic GMP levels occurs only with intact cells and has an EC_{50} near the "low-affinity" agonist equilibrium dissociation constant (K_L) determined by radioligand binding assays. The inhibition of prostaglandin E_1-stimulated [^{3}H]cyclic AMP formation has an EC_{50} close to the value for the "high-affinity" agonist equilibrium dissociation constant (K_H). During sequential subculturing in medium supplemented with newborn bovine serum, the inhibition of [^{3}H]cyclic AMP was maintained, but the [^{3}H]cyclic GMP response declined dramatically, and after 7 subculturings it was essentially absent. The time course for [^{3}H]cyclic GMP formation in a late subculture with an 88% loss of the response was identical with the time course in early subcultures. A normal [^{3}H]cyclic GMP response to bradykinin and histamine was demonstrated to be present in cells that had lost the [^{3}H]cyclic GMP response to carbachol. The EC_{50} and K_D values for the two muscarinic responses and binding sites increased 3- to 4-fold after several subculturings. A 90% loss of low-affinity binding sites was closely correlated with a similar loss of the [^{3}H]cyclic GMP response. High-affinity binding sites did not decline significantly in concentration until the 11th subculture, where the total number of muscarinic sites was only 6% of the earliest subculture. In all subcultures, however, the ability of the muscarinic receptor to decrease [^{3}H]cyclic AMP levels was maintained. These data, which show that the subculturing of N1E-115 cells in medium supplemented with newborn calf serum results in a selective loss of one muscarinic function, strongly support the hypothesis that these cells contain two separate muscarinic receptor-effector systems. One receptor subtype or conformation has a low affinity for the agonist and mediates cyclic GMP formation. The other receptor subtype or conformation has a higher affinity for the agonist and mediates an inhibition of prostaglandin E_1-stimulated cyclic AMP formation.

INTRODUCTION

Murine neuroblastoma cells (clone N1E-115) have been used in our laboratory and those of others as a model system for the study of neuronal receptor function, in particular muscarinic cholinergic receptor mechanisms (1). There are at least five identified muscarinic responses in N1E-115: the stimulation of cyclic GMP levels (2), the inhibition of receptor-linked systems that elevate cyclic AMP (3), the stimulation of the phosphatidylinositol cycle (4), a hyperpolarization of the plasma membrane (5), and the release of arachidonic acid (6). We have previously obtained evidence that multiple binding sites or receptor conformations for muscarinic agonists exist in N1E-115 cells: a high-affinity site (B_H[1]), which mediates the inhibition of adenylate cyclase; and a low-affinity site (B_L), which mediates the stimulation of guanylate cyclase (7). We use "site" or "subtype"

[1] The abbreviations used are: B_H, capacity of the high-affinity agonist binding site; B_L, capacity of the low-affinity agonist binding site; B_{SH}, capacity of the super high-affinity agonist binding site; QNB, quinuclidinyl benzilate; NMS, *N*-methylscopolamine; PGE_1, prostaglandin E_1; K_D, equilibrium dissociation constant; K_H, equilibrium dissociation constant for the high-affinity agonist site; K_L, equilibrium dissociation constant for the low-affinity agonist site; K_N, equilibrium dissociation constant for [^{3}H]NMS; K_Q, equilibrium dissociation constant for [^{3}H]QNB; K_{SH}, equilibrium dissociation constant for the super high-affinity agonist site; R_{SH}, the super high-affinity receptor; R_H, the high-affinity receptor; R_L, the low-affinity receptor.

0026-895X/84/050156-08$02.00/0

interchangeably with "conformation" of the muscarinic receptor, without detailing the molecular mechanism of how various agonists or the antagonist pirenzepine differentiate between these states. The evidence for this linkage of separate responses to different conformations of the muscarinic receptor is provided mainly by (a) the separation of EC_{50} values for the stimulation of the two responses; (b) the correlation of the EC_{50} values for a given response with the K_D for a given binding site; (c) the inhibition by pirenzepine of one response (cyclic GMP elevation) at least 10-fold more potent than the inhibition of the other response (inhibition of cyclic AMP elevation by PGE_1); and (d) the relative efficacy of certain agonists (arecholine, bethanecol, pilocarpine, McN-A343, and oxotremorine) for stimulating one response but not the other (7).[2]

In the course of our studies of the muscarinic receptor, we observed that, with the culturing of the cells in medium supplemented with newborn bovine serum, their responsiveness to muscarinic agonists declined with multiple subculturing. In previous studies, where we grew cells in medium supplemented with fetal bovine serum, stimulation of [^{3}H]cyclic GMP levels greater than 10-fold over basal was maintained for well beyond 10 subculturings. However, with the present medium, after seven subculturings there was an essentially complete loss of the [^{3}H]cyclic GMP response. We have documented this twice during the period covered by this study. Interestingly, the ability of the muscarinic receptor to decrease prostaglandin-stimulated [^{3}H]cyclic AMP levels was fully preserved. This suggested to us that one receptor subtype and/or effector is down-regulated or that a class of N1E-115 cells with only one of the responses is somehow selected for during subculturing. If this were true, it would add support to the hypothesis that each receptor subtype or conformation mediates a separate response.

In the studies reported here, binding experiments and assays for the muscarinic responses were performed with cells for subcultures 9–19, or 11 subcultures from initial subculture after thawing. The data reveal marked functional and binding changes in muscarinic receptors, in particular the low-affinity receptor, of N1E-115 cells during subculturing in medium supplemented with newborn bovine serum. However, the loss of the muscarinic-stimulated [^{3}H]cyclic GMP response occurred without a loss of the histamine H_1- or bradykinin-mediated [^{3}H]cyclic GMP response, and was not attributable to an absence of the guanylate cyclase or a change in the time course of stimulation. These data provide good evidence in support of the hypothesis that there are two muscarinic receptor subtypes or agonist-inducible conformations mediating separate responses in N1E-115 cells.

MATERIALS AND METHODS

Cell culture. The procedures for growth of murine neuroblastoma clone N1E-115 cells are given in detail in previous publications (2–6). Dulbecco's modified Eagle's medium was supplemented wth 10% newborn bovine serum (Grand Island Biological Company, Grand Island, N. Y.). Cells were grown in 75 cm^2 flasks (Falcon Plastics, Cockeysville, Md.) in 20 ml of medium. Cells were fed daily and harvested for assays on days 13–21. Cells were subcultured at a distribution of 1:10 (about 5×10^5 cells/flask). The first subculture after thawing cells stored in liquid N_2 was subculture 9.

Cyclic nucleotide and guanylate cyclase assays. The stimulation of the formation of [^{3}H]cyclic GMP from prelabeled endogenous [^{3}H]GTP by the muscarinic receptor has been described (2). The assay for inhibition of PGE_1-stimulated [^{3}H]cyclic AMP from prelabeled endogenous [^{3}H]ATP by the muscarinic receptor has been described (3). The assay procedure for guanylate cyclase activity has been described (8).

Radioligand binding assays. The equilibrium saturation binding of [^{3}H]QNB (specific activity 26.8 Ci/mmol) and [^{3}H]NMS (specific activity 53.5 Ci/mmol), and the competition between [^{3}H]QNB or [^{3}H]-NMS and carbachol (Sigma Chemical Company, St. Louis, Mo.) were performed. Radioligands were obtained from New England Nuclear Corporation (Boston, Mass.). Binding was generally performed with intact N1E-115 cells in suspension (200,000–500,000 cells/tube) in an isoosmolar physiological phosphate-buffered saline solution (110 mM NaCl/5.3 mM KCl/1.8 mM $CaCl_2$/1.0 mM $MgCl_2$/25 mM glucose/25 mM Na_2HPO_4; pH adjusted to 7.35; osmolality adjusted to 340 ± 5 mOsm with sucrose). Each ligand concentration was assayed in triplicate in 2 ml total volume. Assay temperature was maintained at 15° to prevent desensitization by carbachol during the 75-min assay (7, 9). Bound [^{3}H]QNB or [^{3}H]NMS was separated from free ligand by vacuum filtration over Whatman GF/B filters (Whatman, England). The filters were then washed four times with 4 ml each of ice-cold 0.9% NaCl solution. Filters were solubilized for 2 hr in Safety-Solve (Research Products International, Elk Grove Village, Ill.), and radioactivity was measured by liquid scintillation counting. The maximal number of binding sites (B_{max}) and the K_Q for [^{3}H]QNB were determined by Scatchard analysis (10) of the saturation [^{3}H]QNB binding. [^{3}H]QNB binding was monophasic, and the value of K_Q was 0.38 ± 0.02 nM (n = 13) at this temperature and did not vary with subculture. The K_N for [^{3}H]NMS, obtained by saturation binding, was 0.19 ± 0.02 nM (n = 7) at this temperature; [^{3}H]NMS binding also was monophasic, and the K_N did not vary with subculture. The parameter K_Q or K_N was fixed as a constant in the independent two-site or three-site (as appropriate) ^{3}H-labeled antagonist-agonist competition model used in computer analysis of agonist binding sites. This iterative nonlinear analysis [described in detail in McKinney and Coyle (11)] provides estimates of the dissociation constants (K_H, K_L, K_{SH}) for carbachol bound to the high-, low-, and super high-affinity agonist sites and the capacities (B_H, B_L, and B_{SH}) for these three sites. The concentration of [^{3}H]QNB or [^{3}H]NMS used in competition assays was 0.2–0.4 nM.

For determination of nonspecific binding (which can include uptake of [^{3}H]QNB), the amount of [^{3}H]QNB or [^{3}H]NMS in the presence of 10 mM carbachol was usually used. One micromolar atropine, in preliminary experiments, occasionally revealed about 10–15% more [^{3}H]QNB "sites," conceivably due to the uptake of [^{3}H]QNB and atropine. This point was examined with the use of 1 μM NMS, an impermeant muscarinic ligand. NMS and carbachol competed for a similar quantity of [^{3}H]QNB binding, whereas atropine competed for about 10% more of the [^{3}H]QNB binding whether or not the cells were homogenized. Therefore, the asymptomatic value of [^{3}H]QNB bound at 10 mM carbachol was used as an estimate of nonspecific binding and uptake of [^{3}H]QNB if 1 μM atropine, which was also in some tubes for comparison, competed for more [^{3}H]QNB binding.

We have observed that there is somewhat less specific [^{3}H]QNB binding in homogenates than in intact cells. To ensure that the greater [^{3}H]QNB binding with intact cells was not due to uptake, we compared specific binding in intact cells with that in cells sonicated just before filtration (to allow washout of uptake of radioligand). Calculated maximal specific binding was the same in both cases and indicated that the binding assay with intact cells reflected true specific receptor binding. Specific binding was less in homogenates of these cells (we have observed up to 40% loss of sites in fresh whole homogenates of N1E-

[2] M. McKinney, S. Stenstrom, and E. Richelson, manuscript in preparation.

115 cells in 30 min). Our data indicate that an instability of the muscarinic receptor accounts for less binding in homogenates.[3] A comparison of agonist equilibrium dissociation constants (K_H and K_L) and the proportion of sites measured in intact cells and in homogenates under the same conditions (buffer composition and temperature) indicated that they were similar. It therefore appeared valid and expedient to perform binding to muscarinic receptors in intact cells. Our data are consistent with the concept that most, if not all, [^{3}H]QNB binding sites are located on the surface of the cell, and thus are accessible to NMS, atropine, and carbachol in the medium. We further examined this postulate in several experiments by analyzing the competition of [^{3}H]NMS and carbachol. Nonspecific binding in intact cells with this radioligand is very low (under the conditions of our assay, nonspecific binding is 10% or less of total binding), as it is not sequestered inside the cell. The capacities and equilibrium dissociation constants for the high- and low-affinity agonist sites obtained in this manner were essentially identical ($n = 5$) with those obtained by [^{3}H]QNB/carbachol binding; therefore, [^{3}H]NMS/carbachol data were combined with [^{3}H]QNB/carbachol data.

A third agonist site, the super high-affinity site (R_{SH}), was sometimes observed (in five experiments) with intact cells, but only when [^{3}H]QNB was used as the radioligand and when many data allowed for a statistical demonstration [by the F-ratio test (12)]. When this site was observed, it had a dissociation constant (K_{SH}) of 5 ± 3 nM and a capacity of 18 ± 3%; it is possibly analogous to the site in rat brain (13). In Fig. 6, to show evidence for all of the agonist binding sites in N1E-115 cells, we show two subcultures that possessed this site as well as the other two sites. For the purpose of this paper, however, we discuss in detail only the two major sites (R_H and R_L). Generally, 16–32 pairs of data (free carbachol concentration and amount of [^{3}H]QNB or [^{3}H]NMS bound) were analyzed to evaluate K_H, K_L, B_H, and B_L. Constraints for non-negative values were applied to estimates of these parameters.

Statistical tests. For data reported as arithmetic means (± standard errors) of data obtained in individual experiments, the statistical test used was the unpaired t-test. Parameter values reported as significant were for $p < 0.05$. The computer program used for binding analysis (11) provides an estimate of the standard error of parameter values; for some of the data of Fig. 6, these are reported. For testing the significance of fit of two- versus three-site binding models, the F-ratio method was used (12).

RESULTS

Observations on cell growth. In the early subcultures in newborn bovine serum (subcultures 9–11), the cells were large and grew in clumps loosely attached to the bottoms of the flasks. With increasing subcultures, the cells diminished in size and attached more uniformly and firmly to the bottoms. The number of cells per flask at confluence increased progressively with subculturing. In 11 subculturings, the number of cells per flask (assayed at confluence) nearly tripled. Cell number per flask increased from 3.4 ± 0.1 × 10^6 (subculture 9, n = 3) to 9.6 ± 1.4 × 10^6 (subculture 19, n = 2).

Changes in cyclic nucleotide responses to muscarinic stimulation. The data reported in this paper were compiled from two separate series of subculturings that began with subculture 9. In both series, the [^{3}H]cyclic GMP response was observed to decline progressively until at subculture 15 or 16 the response was barely detectable. We demonstrate in Fig. 1 that the response was absent in the same experiment in which there was a normal response in an earlier subculture (a positive control).

[3] M. McKinney, S. Stenstrom, and E. Richelson, manuscript in preparation.

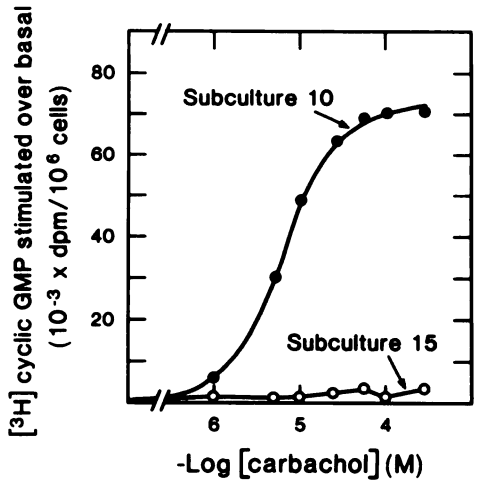

FIG. 1. *Loss of the muscarinic receptor-mediated [^{3}H]cyclic GMP response*

An early subculture (*10*) and a late subculture (*15*) were assayed in the same concentration-response experiment using the method of prelabeling [^{3}H]GTP stores with [^{3}H]guanine.

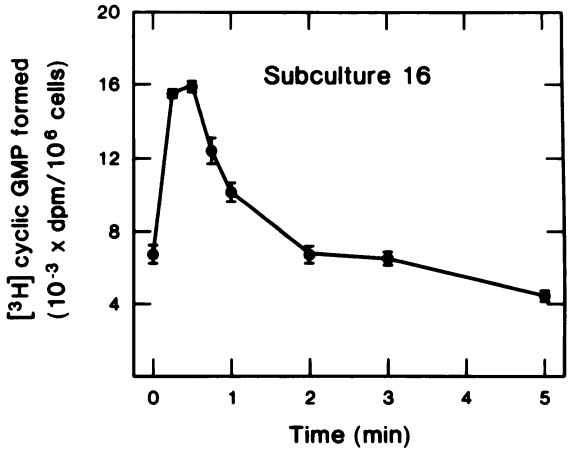

FIG. 2. *Time course of [^{3}H]cyclic GMP formation during stimulation with 1 mM carbachol in a late subculture (16)*

The maximal muscarinic receptor-mediated response occurs at the assay time point of 30 sec and is only 12% of the response at subculture 9.

This experiment rules out a defect in the execution of the experimental protocol.

The time course for receptor-mediated [^{3}H]cyclic GMP formation is transient, and our assays are routinely stopped at 30 sec (the peak of the response). If there were a shift in the peak of [^{3}H]cyclic GMP formation, this might account for the apparent loss of the response in later cultures. However, cells of subculture 16 (from the first series of subculturings), in which only 12% of the muscarinic receptor-mediated [^{3}H]cyclic GMP response remained, had a time course of formation (Fig. 2) identical with those previously published (14), with a peak at the assay point of 30 sec.

The induced change was specific for muscarinic receptor-mediated [^{3}H]cyclic GMP response, since cells that were unresponsive to carbachol were responsive to both histamine (Fig. 3A) and bradykinin (Fig. 3B). Histamine mediates [^{3}H]cyclic GMP formation in N1E-115 cells via histamine H_1 receptors (15); bradykinin receptors have

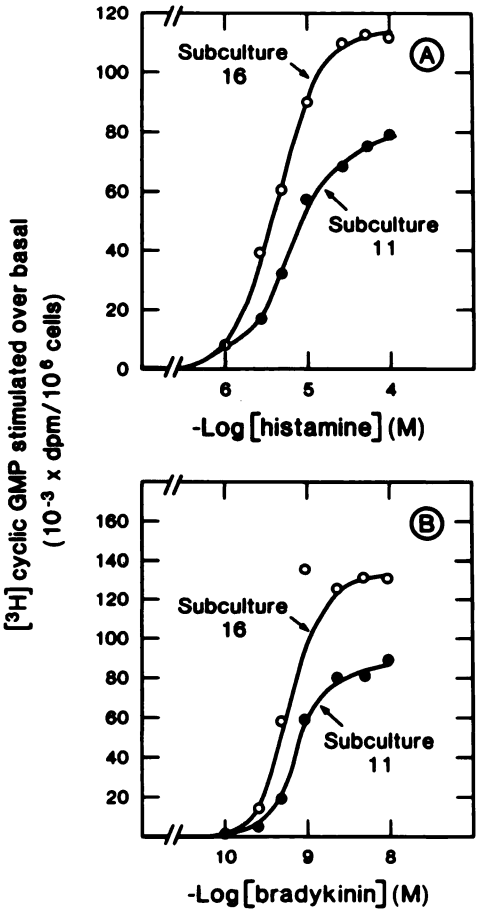

FIG. 3. *Retention of the [³H]cyclic GMP response to histamine and bradykinin in cells of subculture 16, which had lost the muscarinic receptor-mediated [³H]cyclic GMP response*

A. Concentration-response for histamine-[³H]cyclic GMP. B. Concentration-response for bradykinin-[³H]cyclic GMP. Basal levels of [³H]cyclic GMP have been subtracted. Cells of subcultures 11 and 16 were assayed in the same experiment.

recently been characterized in these cells (16). In addition, the ability of subculture 16 cells to increase [³H] cyclic GMP levels in response to these two agents exceeded that of control (subculture 11) cells in the same assay. This result appears to indicate that (a) the labeling of the intracellular pool of [³H]GTP by [³H]guanine in cells that were unresponsive to carbachol was similar to that in cells which had the full response; (b) this pool of [³H]GTP could be converted into [³H]cyclic GMP by receptor activation, ruling out a general loss of all receptor-effector function; and (c) the induced change in the cell is specific for muscarinic receptors.

That the guanylate cyclase enzyme was present and active in the cells of higher subculture was shown additionally by direct assay of the enzyme velocity in homogenates. For cells of subculture 15, the velocity was 19 ± 1 nmol/hr·10^6 cells equivalent, a value nearly identical with the velocity measured in cells of subculture 10 (21 ± 1 nmol/hr·10^6 cells equivalent). Enzymes of both preparations were activated by 500 μM sodium nitroprusside to 36 ± 1 and 43 ± 8 nmol/hr·10^6 cells equivalent, respectively.

A plot of the maximal [³H]cyclic GMP response obtained with carbachol and the corresponding basal levels in each subculture studied appears in Fig. 4A. The basal levels of [³H]cyclic GMP did not vary with subculture; the average basal level over all subcultures assayed was 4500 ± 300 dpm/10^6 cells ($n = 61$). However, the maximal [³H]cyclic GMP levels stimulated with carbachol declined progressively until they reached a very low magnitude in subculture 15 or 16. In some assays of these cultures there were detectable responses (as in Fig. 2); in other assays no responses were measurable (as in Fig. 1). A further test of subculture 19 also was negative. These results have been obtained by several different workers in the laboratory.

In contrast, the ability of carbachol to inhibit PGE_1-stimulated [³H]cyclic AMP formation was preserved even to subculture 19 (Fig. 4B). Carbachol was able to decrease PGE_1 (1 μM)-stimulated [³H]cyclic AMP levels by 35–65% of maximum in all subcultures assayed. Basal

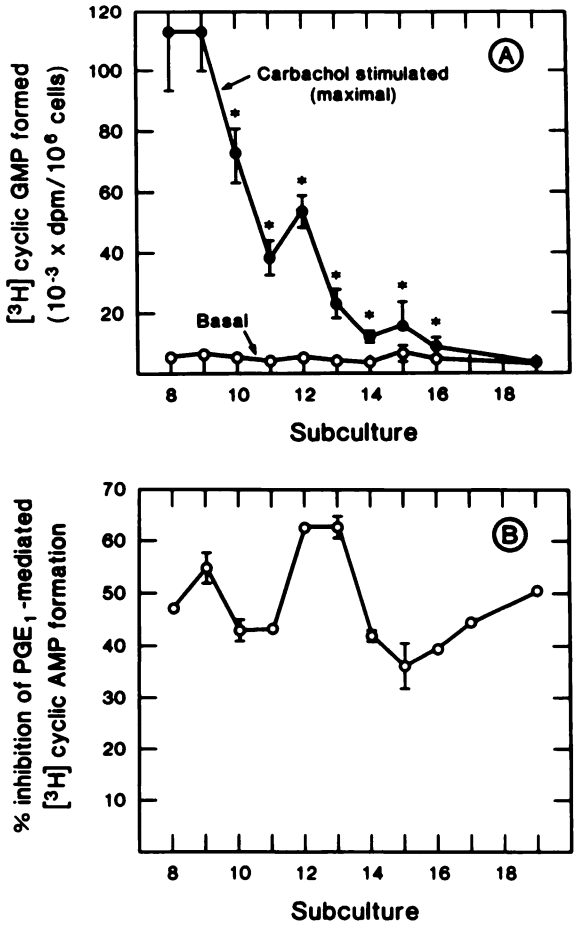

FIG. 4. *Variation in the degree of response to muscarinic stimulation during subculturing*

A. The decrease in the maximal muscarinic receptor stimulation of [³H]cyclic GMP and constancy of basal levels of [³H]cyclic GMP in various subcultures of N1E-115 cells. B. Retention of the muscarinic inhibition (maximal levels) of PGE_1-stimulated [³H]cyclic AMP formation from prelabeled [³H]ATP stores. The concentration of PGE_1 used in these assays was 1 μM (a maximal stimulation). Values are averages, and standard errors are shown as *vertical bars* where multiple assays were performed. Bars for basal [³H]cyclic GMP values lie within the *symbols*. *Asterisks* indicate $p < 0.05$ for comparison with subculture 9.

[^{3}H]cyclic AMP levels did not vary significantly with subculture; the average value over all subcultures was 5000 ± 500 dpm/10^6 cells (n = 32). The ability of 1 μM PGE_1 to stimulate [^{3}H]cyclic AMP maximally was somewhat variable but did not vary consistently with subculturing, except that it tended to be somewhat lower in later subcultures. In cells of subculture 8, the level of [^{3}H]cyclic AMP in the presence of 1 μM PGE_1 was 120,000 ± 20,000 dpm/10^6 cells. In subcultures 17 and 19, for example, the levels of [^{3}H]cyclic AMP formed in the presence of PGE_1 were 61,000 and 62,000 dpm/10^6 cells, respectively. The average maximal level of [^{3}H]cyclic AMP stimulated by 1 μM PGE_1 for all subcultures was 110,000 ± 20,000 (n = 32).

The effective concentration for 50% of maximal response (EC_{50}) for each muscarinic response increased with subculturing. In later subcultures, the EC_{50} for the [^{3}H]cyclic GMP response was increased about 6-fold, and the EC_{50} for [^{3}H]cyclic AMP inhibition was 4 times greater than that for cells of subculture 9 (Fig. 5A and B, respectively). These data were highly significant statistically ($p < 0.001$ for the difference between cells of the latest subculture tested and those of subculture 9).

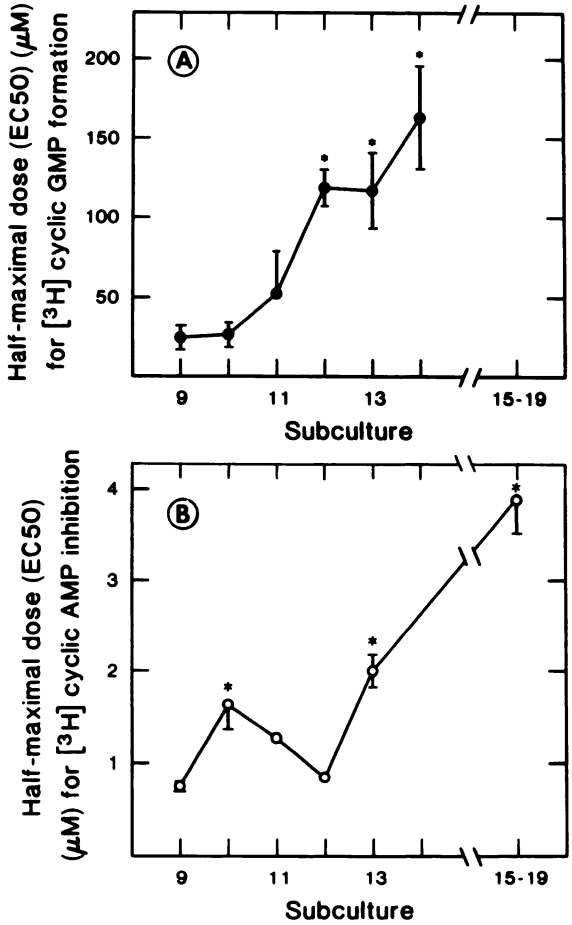

FIG. 5. *Increases in EC_{50} values for muscarinic responses in N1E-115 cells during subculturing*

A. EC_{50} values for [^{3}H]cyclic GMP stimulation. B. EC_{50} values for [^{3}H]cyclic AMP inhibition. Values are averages ± standard error. *Asterisks* indicate significance ($p < 0.05$) in comparison with subculture 9.

The EC_{50} for the [^{3}H]cyclic GMP response did not begin to change until after subculture 10, whereas the EC_{50} for the [^{3}H]cyclic AMP response did not increase much until after subculture 12.

Changes in muscarinic receptor binding during subculturing. For the purpose of studying certain aspects of receptor function, such as regulation, it is necessary to perform binding experiments with intact cells. Preliminary binding experiments were performed with intact N1E-115 cells to rule out any potential problem with uptake of the ligands (see Materials and Methods). Because the desensitization and down-regulation of muscarinic receptors in intact N1E-115 cells will occur in the time frame of a binding experiment if there is an agonist present at temperatures above 22°, the binding experiment with intact cells must be performed below this temperature (7, 9). Several experiments were performed with homogenates of N1E-115 cells at 37° to verify that agonist binding affinities at this temperature were comparable to those obtained with intact cells at 15°. At 37° in homogenates, the super high-affinity site was not consistently observed. However, the high- and low-affinity agonist sites were always present in proportions and with binding constants similar to those at 15°. The low-affinity constant (K_L) was 60 ± 20 μM at 37° (n = 4) and was comparable to the K_L at 15°, averaged for cells over the same subculture numbers (10–14), 40 ± 10 μM (n = 12). The value of K_H at 37° was 1.1 ± 0.4 μM (n = 4) and at 15° it was 1.1 ± 0.2 (n = 12). These values for carbachol were similar to those reported previously for homogenates of N1E-115 cells at 30° (17) and were not significantly different from values of K_H and K_L observed with binding to a homogenate of rat cortex at 25° (11). The equilibrium dissociation constant for [^{3}H]QNB, however, was markedly affected by temperature; it was 10-fold greater at 15° (0.38 ± 0.02 nM, n = 12) than at 37° (0.043 ± 0.006 nM, n = 7), and this value (K_Q) was used in the analytical equation as appropriate. Since temperature did not seem to be a major factor in agonist binding, we chose to perform binding with intact cells at 15°, because assays for agonist binding at 37° would be complicated by desensitization, and the values of the parameters would likely not represent those in naive cells (9).

Representative curves for competition between [^{3}H]QNB and carbachol at 15° for cells of subcultures 9, 14, and 19 (Fig. 6) demonstrate marked decreases in binding after multiple subculturing. A large number of data points was used in order to reveal clearly the multiphasic nature of the competition profile. Additionally, binding data that demonstrate the presence of the third agonist site (R_{SH}) are shown. In the data shown for cells of subcultures 9 and 14, there were three sites present (R_{SH}, R_H, and R_L). For the data of subculture 9, for which the three-site model fit significantly better than did a two-site model ($p < 0.001$), the capacities of these sites were 19 ± 4% (B_{SH}), 17 ± 3% (B_H), and 64 ± 3% (B_L) with equilibrium constants of 0.3 ± 0.2 nM (K_{SH}), 0.10 ± 0.07 μM (K_H), and 26 ± 4 μM (K_L).

Cells of subculture 14 had very few low-affinity sites (7% of the level of cells of subculture 9, as shown in Fig. 6), whereas these cells had high-affinity and super-high

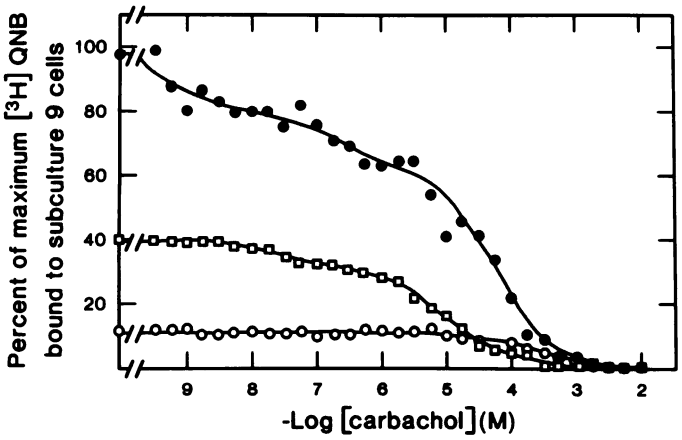

FIG. 6. *[³H]QNB-carbachol competition curves at 15° for subculture 9 (●), subculture 14 (□), and subculture 19 (○), demonstrating decreased muscarinic binding with subculturing*

Thirty-two concentrations of carbachol were assayed in triplicate in competition with a fixed concentration of [³H]QNB. Subculture 19 cells were homogenized in order to reduce nonspecific binding. Data were normalized and expressed as percentages of the maximal binding of subculture 9. The smooth curves represent the computer-generated best fit to a three-site model, except that subculture 19 cells gave only one site in the analysis. Parameter values are given in the text.

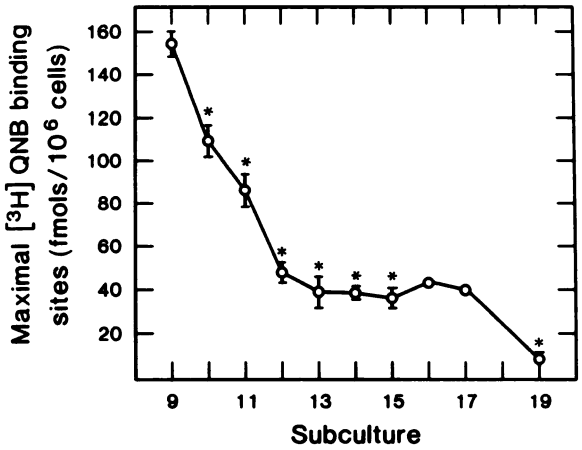

FIG. 7. *Decrease in the total number (B_{max}) of [³H]QNB-labeled or [³H]NMS-labeled muscarinic receptor sites during subculturing of N1E-115 cells in newborn bovine serum*

Data marked with *asterisks* indicate that they were significantly different from those of subculture 9.

affinity sites at about the same level as in cells of subculture 9. For cells of subculture 19, there was only one class of sites with a K_D of 16 μM. Scatchard plots of [³H]QNB binding also demonstrated a reduction in maximal binding (data not shown). Thus, the changes in response discussed above were paralleled by changes in binding, and therefore a detailed study of the receptor capacities and affinities was pursued.

The maximal binding (total of all sites) of muscarinic receptors in N1E-115 cells decreased in the first four subcultures to a level of only about 25% of the initial subculture (Fig. 7). At subculture 19, long after the loss of the [³H]cyclic GMP response, the level of receptors was decreased to only about 6% of that in cells of subculture 9. When receptor subtypes were assayed by [³H]QNB-carbachol or [³H]NMS-carbachol competition in various subcultures, it was observed that decreases in the concentration of low-affinity receptors accounted for the loss of receptor binding (Fig. 8A). High-affinity receptors were not significantly lower in cells of subcultures 12–17 as compared with cells of subcultures 9–11, but by subculture 19 the high-affinity receptors had decreased markedly in number (Fig. 8B). However, parallel assays of sister cells of subculture 19 indicated that the ability of carbachol to inhibit PGE_1-stimulated [³H]cyclic AMP formation was not impaired (see Fig. 4B). An excellent correlation ($r = 0.99$) between the maximum carbachol-stimulated [³H]cyclic GMP levels and the concentration of the low-affinity receptor was obtained (Fig. 9), a result strongly suggestive of a relationship between the two.

There was an early phase (subcultures 9 and 10 for the [³H]cyclic GMP response and subcultures 9–12 for the [³H]cyclic AMP response) in which the EC_{50} values did not increase much (Fig. 5). This suggested that a correlation between EC_{50} values and binding constants might be observable if the data were divided between an early phase (where cells have not changed much) and a late

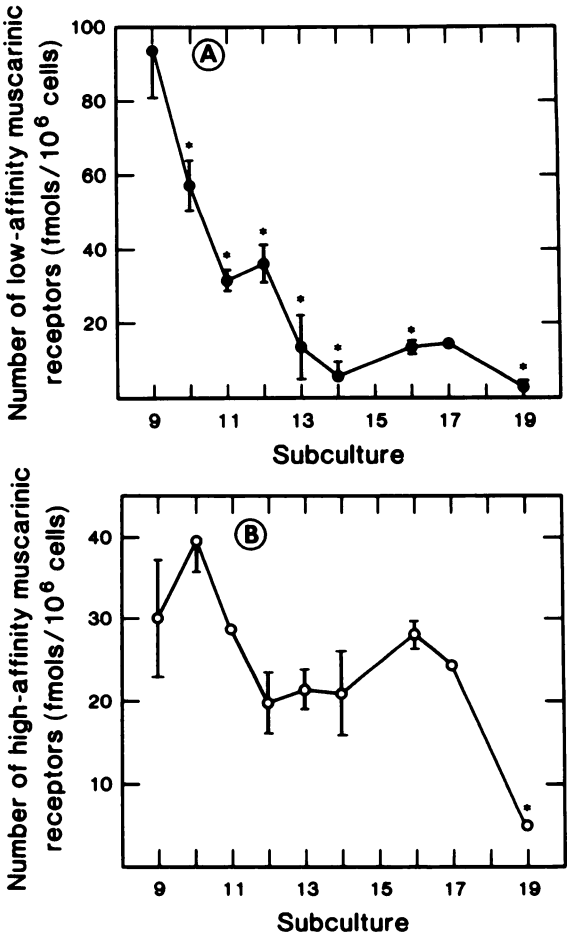

FIG. 8. *Changes in the concentration of low-affinity agonist sites (A) and in the concentration of high-affinity agonist sites (B) during subculturing of N1E-115 cells in medium supplemented with newborn bovine serum*

The data are averages ± range or standard error for two to eight assays at each point (except for a single estimate at subculture 17). *Asterisks* indicate data significantly different from the value at subculture 9.

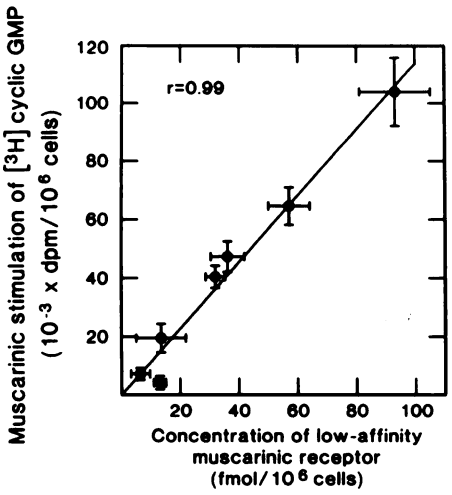

FIG. 9. *Correlation between the concentration of low-affinity muscarinic receptors and the maximal levels of muscarinic receptor-stimulated [^{3}H]cyclic GMP*

Data from Fig. 4A and Fig. 8A (subcultures 9–14 and 16) were used. Basal levels of [^{3}H]cyclic GMP were subtracted from maximal levels shown in Fig. 4A to obtain the amount of [^{3}H]cyclic GMP formed in response to receptor activation. Linear regression by the method of least squares indicated a relationship between the two parameters (the plotted line) with a correlation coefficient ($r = 0.99$).

TABLE 1

Correlation between EC_{50} values for cyclic nucleotide responses and K_D values for muscarinic receptor subtypes in intact N1E-115 cells

Data for [^{3}H]cyclic GMP and [^{3}H]cyclic AMP responses were obtained at 37° as described under Materials and Methods. Values of dissociation constants K_H and K_L were obtained by computer-aided analysis of [^{3}H]QNB-carbachol or [^{3}H]NMS-carbachol binding performed at 15°. In a given block the EC_{50} and K_D values are not significantly different. The EC_{50} values for both responses in the late phase are significantly different from the respective EC_{50} values in the early phase ($p < 0.001$). The K_D values in the late phase are significantly different from those in the early phase ($p < 0.025$ for K_H; $p < 0.001$ for K_L).

Parameter	Early phase	Late phase
	Subcultures 9–10	Subcultures 11–14
EC_{50} for cyclic GMP stimulation	24 ± 3 ($n = 12$)	110 ± 10 ($n = 10$)
K_L	18 ± 2 ($n = 8$)	70 ± 10 ($n = 6$)
	Subcultures 9–12	Subcultures 14–19
EC_{50} for cyclic AMP inhibition	1.3 ± 0.2 ($n = 9$)	3.6 ± 0.4 ($n = 10$)
K_H	0.9 ± 0.1 ($n = 12$)	2.4 ± 0.6 ($n = 7$)

phase (where receptor changes have occurred). This comparison is shown in Table 1. The increase in EC_{50} values for both responses was accompanied by similar increases in binding constants. The EC_{50} for cyclic GMP stimulation was not different from the K_L in either phase. The EC_{50} for cyclic AMP inhibition was also similar to the K_H in both phases. There was a tendency for the K_D to be lower than the respective EC_{50}. The increases in EC_{50} values and binding constants from early to late phase were statistically significant and provide evidence in support of the hypothesis that the low-affinity receptor is coupled to the stimulation of cyclic GMP whereas the high-affinity receptor is coupled to the inhibition of receptor-linked adenylate cyclase stimulation.

DISCUSSION

In this study we have demonstrated decreases in muscarinic function in N1E-115 cells during subculturing of these cells in newborn bovine serum. Essentially complete loss of the [^{3}H]cyclic GMP response occurred in seven subculturings in association with a 90% decrement in the number of low-affinity muscarinic receptors. High-affinity receptors were maintained through 10 subculturings at nearly control levels, and the ability of the muscarinic receptor to inhibit the PGE_1-mediated [^{3}H]cyclic AMP response was not lost even after 11 subculturings.

The correlation during subculturing between the EC_{50} for [^{3}H]cyclic GMP stimulation and K_L is one indication that the [^{3}H]cyclic GMP response is mediated by the low-affinity receptor. The correlation between K_H and the EC_{50} for the [^{3}H]cyclic AMP response is also suggestive of a relationship between R_H and the inhibition of the PGE_1-mediated [^{3}H]cyclic AMP response, as has been noted previously (1, 7). Other data that support these concepts are summarized under Introduction.

There are several aspects of the data that suggest that the decreased [^{3}H]cyclic GMP response is due to a specific change in one subtype of muscarinic receptor. The decline in carbachol's maximal stimulation of the response is closely paralleled by the decline in B_L and not B_H. Assay of guanylate cyclase activity indicated that this enzyme was present and as active in subculture 15 as in earlier subcultures. The time course of [^{3}H]cyclic GMP stimulation was the same in subculture 16 as in earlier cultures. The retention of the ability of bradykinin and histamine to stimulate the [^{3}H]cyclic GMP response indicates that no major nonspecific changes occurred either in the plasma membrane or in the receptor-stimulated [^{3}H]GTP pool. Therefore, specific changes in the low-affinity muscarinic receptor and/or effector are likely to account for the loss of the [^{3}H]cyclic GMP response. A possibility that has not been completely ruled out is the presence of higher levels of a cyclic GMP phosphodiesterase activity in the later subcultures, which might prevent elevation of [^{3}H]cyclic GMP levels. The positive results with bradykinin and histamine are evidence against this possibility, however, unless there is hypothesized to be a cholinergically stimulated phosphodiesterase activity that appears or is increased in the later subcultures. The close correlation between the stimulation of [^{3}H]cyclic GMP by carbachol and the concentration of R_L, besides indicating a relationship between the two, would also indicate that the primary event occurring during subculturing is the disappearance of the low-affinity muscarinic receptor, or the loss of the class of receptors in which the agonist can induce the low-affinity conformation.

We cannot yet provide an apparent functional correlate for the super high-affinity agonist site. The fact that the super high-affinity site has so far been observable only in intact cells with [^{3}H]QNB suggests that it may

be denatured, degraded, or converted into another type of site upon homogenization or that it is a peculiarity of [^{3}H]QNB-carbachol competition. A similar site was observed in rat brain membranes with another [^{3}H]antagonist (13).

Shifrin and Klein (18) also have noted a decline in the number of muscarinic receptors on N1E-115 cells with numerous subculturings in medium supplemented with fetal calf serum. Thus it appears that loss of receptors could occur with either supplement, but apparently much more rapidly with newborn bovine serum.

Our results with newborn bovine serum have led us to begin controlled experiments to compare muscarinic responses in cells cultured in parallel for an extended number of subculturings in medium supplemented with fetal bovine serum and in medium supplemented with newborn bovine serum. We have found that the use of medium supplemented with fetal bovine serum will not cause the muscarinic receptor-guanylate cyclase system to recover the ability to mediate the response and return to a "normal" number of low-affinity sites, indicating that the induced change in function is of a permanent nature.

REFERENCES

1. McKinney, M., and E. Richelson. The coupling of the neuronal muscarinic receptor to responses. *Annu. Rev. Pharmacol. Toxicol.* **24**:121–146 (1984).
2. Richelson, E., F. G. Prendergast, and S. Divinetz-Romero. Muscarinic receptor-mediated cyclic GMP formation by cultured nerve cells: ionic dependence and effects of local anesthetics. *Biochem. Pharmacol.* **27**:2039–2048 (1978).
3. Stenstrom, S., and E. Richelson. Acute effect of ethanol on prostaglandin E_1-mediated cyclic AMP formation by a murine neuroblastoma clone. *J. Pharmacol. Exp. Ther.* **221**:334–341 (1982).
4. Cohen, N. M., D. M. Schmidt, R. C. McGlennen, and W. L. Klein. Receptor-mediated increases in phosphatidylinositol turnover in neuron-like cell lines. *J. Neurochem.* **40**:547–554 (1983).
5. Wastek, G. J., J. R. Lopes, and E. Richelson. Demonstration of a muscarinic receptor-mediated cyclic GMP-dependent hyperpolarization of the membrane potential of mouse neuroblastoma cells using [^{3}H]tetraphenylphosphonium. *Mol. Pharmacol.* **19**:15–20 (1981).
6. Snider, R. M., M. McKinney, C. Forray, and E. Richelson. Neurotransmitter receptors mediate cyclic GMP formation by involvement of phospholipase A_2 and arachidonic acid metabolites. *Proc. Natl. Acad. Sci. U. S. A.* **81**: 3905–3909 (1984).
7. McKinney, M., S. Stenstrom, and E. Richelson. Effect of short-term desensitization on muscarinic receptor binding in intact murine neuroblastoma cells (clone N1E-115). *Abstr. Soc. Neurosci.* **9**:581 (1983).
8. El-Fakahany, E., and E. Richelson. Regulation of muscarinic receptor-mediated cyclic GMP synthesis by cultured mouse neuroblastoma cells. *J. Neurochem.* **35**:941–948 (1980).
9. El-Fakahany, E., and E. Richelson. Temperature dependence of muscarinic acetylcholine receptor activation, desensitization, and resensitization. *J. Neurochem.* **34**:1288–1295 (1980).
10. Scatchard, G. The attraction of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**:660 (1949).
11. McKinney, M., and J. T. Coyle. Regulation of neocortical muscarinic receptors: effects of drug treatment and lesions. *J. Neurosci.* **2**:97–105 (1982).
12. De Lean, 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 (1981).
13. Birdsall, N. J. M., E. C. Hulme, and A. Burgen. The character of the muscarinic receptors in different regions of the rat brain. *Proc. R. Soc. Lond. B Biol. Sci.* **207**:1–2 (1980).
14. Matsuzawa, H., and M. Nirenberg. Receptor-mediated shifts in cGMP and cAMP levels in neuroblastoma cells. *Proc. Natl. Acad. Sci. U. S. A.* **72**:3472–3476 (1975).
15. Richelson, E. Histamine H_1 receptor-mediated guanosine 3′,5′-monophosphate formation by cultured mouse neuroblastoma cells. *Science (Wash. D. C.)* **201**:69–71 (1978).
16. Snider, R. M., and E. Richelson. Bradykinin receptor-mediated cyclic GMP formation in a nerve cell population (murine neuroblastoma clone N1E-115). *J. Neurochem.*, in press (1984).
17. Strange, P. G., N. J. M. Birdsall, and A. S. V. Burgen. Ligand binding properties of the muscarinic acetylcholine receptor in mouse neuroblastoma cells. *Biochem. J.* **172**:495–501 (1978).
18. Shifrin, G. S., and W. L. Klein. Regulation of muscarinic acetylcholine receptor concentration in cloned neuroblastoma cells. *J. Neurochem.* **34**:993–999 (1980).

Send reprint requests to: Dr. Elliott Richelson, Department of Pharmacology, Mayo Foundation, Rochester, Minn. 55905.

This research was supported by the Mayo Foundation and by United States Public Health Service Grants AA 4443, MH 27692, and AM 07147.