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

Mediation of Separate Responses by High Affinity and Low Affinity Agonist-Receptor Conformations

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SUMMARY

Murine neuroblastoma cells (clone N1E-115) possess muscarinic receptors that mediate multiple responses, including the elevation of cyclic GMP levels and the inhibition of receptor-mediated increases in cyclic AMP. Evidence is presented showing that two muscarinic agonist-receptor conformations in N1E-115 cells each separately mediate a cyclic nucleotide response. Pirenzepine inhibited the [^{3}H]cyclic GMP response to carbachol with a K_D value of approximately 6 nM, whereas it inhibited the ability of carbachol to reduce prostaglandin E_1-mediated elevations in [^{3}H]cyclic AMP levels with a K_D value of 93 nM, thus differentiating between two classes of receptors involved in these responses. Ten muscarinic agonists were studied for their ability to mediate the two cyclic nucleotide responses. Six were as effective as acetylcholine in the reduction of [^{3}H]cyclic AMP levels, but only two were as effective as acetylcholine in elevating [^{3}H]cyclic GMP levels. Four agonists (arecoline, pilocarpine, oxotremorine, and McN-A343) were ineffective in increasing [^{3}H]cyclic GMP levels. These four agonists and bethanecol, which could increase [^{3}H]cyclic GMP levels only 18% as well as acetylcholine, behaved as competitive antagonists in this response to carbachol. These partial agonists, in contrast to carbachol, bound to only one class of muscarinic sites in N1E-115 cells with equilibrium dissociation constants determined by competition binding assays which agreed well with their respective EC_{50} values for their effect on [^{3}H]cyclic AMP levels. The equilibrium dissociation constants for the partial agonists determined by their inhibition of carbachol in the [^{3}H]cyclic GMP response also agreed well with their respective EC_{50} values for mediating the [^{3}H]cyclic AMP response. Thus, the partial agonists bound to the same receptors at which carbachol mediated [^{3}H]cyclic GMP formation, but with K_D values about the same as their respective EC_{50} values for inhibition of prostaglandin E_1-mediated [^{3}H]cyclic AMP increases. The full agonists acetylcholine and methacholine, like carbachol, bound to two sites in N1E-115 cells. For the six agonists able to stimulate both responses at least to some degree, the ratio of their potencies at each response correlated with their respective efficacies at each response but with much more dependence in the [^{3}H]cyclic GMP response. It is proposed that the low affinity agonist-receptor conformation, inducible by full agonists, activates the effector for the cyclic GMP response and its formation is inhibited by pirenzepine with high potency; and the high affinity agonist-receptor conformation, inducible by both full and partial agonists, activates the effector for the cyclic AMP reduction and is inhibited by pirenzepine with lower potency.

INTRODUCTION

Murine neuroblastoma cells (clone N1E-115) provide an excellent system for the study of muscarinic receptor function (1, 2). In these cells, muscarinic receptors mediate increased cyclic GMP levels, decreased cyclic AMP levels, phospholipid turnover, arachidonic acid metabolism, and hyperpolarization of the plasma membrane. Several of these responses are also mediated by histamine (3), bradykinin (4), thrombin (55, neurotensin (6), and other receptors in N1E-115 cells. We have recently published data supporting a hypothesis that the effector system for receptors increasing cyclic GMP levels may be a system involving metabolism of arachidonic acid (7). The muscarinic receptor of N1E-115 cells is particularly interesting because it appears unique in its ability

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to induce both an increase in cyclic GMP levels and a decrease in cyclic AMP levels within the same cell. Multiple muscarinic receptor-binding sites are the rule rather than the exception, and they have been demonstrated to be present in N1E-115 cells (8–10). The simultaneous presence of multiple muscarinic responses and binding sites in the same cell potentially can help to clarify the mechanisms of muscarinic receptor coupling in neural tissue. It has been supposed (11, 12) that the well established heterogeneity of binding sites for agonists or the antagonist pirenzepine is somehow related to the multiplicity of responses observed in various tissues. Indeed, binding site K_D[1] or K_i values often correlate to some degree with the EC_{50} for stimulation or inhibition of certain responses. But the presence of multiple cell types, the complexity of whole-animal studies, or the presence of "spare" receptors limits the degree to which a rigorous interpretation can be made. We have avoided many of these problems by using N1E-115 cells and by combining physiological response data with binding data obtained in parallel.

Our recent study (10) indicated that one of the two cyclic nucleotide responses in N1E-115 cells ([^{3}H]cyclic GMP formation) is lost after multiple subculturing and there occurs in close parallel a loss of the low affinity carbachol-binding site. The high affinity carbachol site is retained, as is the [^{3}H]cyclic AMP response (inhibition of prostaglandin receptor-mediated increases). The results suggest that the receptor "subtypes" are distinctly different (i.e., independent sites and not cooperative); however, a number of receptor mechanisms not involving distinct subclasses of muscarinic receptors might still be invoked to explain the data. We use "sites" or "conformations" interchangeably without reflection on the underlying molecular nature of the receptor giving rise to the multiplicity in binding or response. Here, we present data that provide further insight into muscarinic receptor coupling in N1E-115 cells. The "nonclassical" antagonist pirenzepine was used to obtain strong evidence for the hypothesis that both responses were mediated by different binding sites. Binding studies with intact cells or homogenates also demonstrated the presence of multiple sites for carbachol, for other full agonists (i.e., "full" in that they mediated both responses as well as acetylcholine) and for pirenzepine. A group of 10 muscarinic agonists was studied for structure-activity relationships in mediating the two cyclic nucleotide responses. These drugs bound to all the [^{3}H]NMS-labeled muscarinic receptors, but there were differences in the potencies and efficacies at the two different responses. The data suggest that full agonists can induce or differentiate between two conformations of the muscarinic receptor: the high affinity site or conformation, which mediates [^{3}H]cyclic AMP inhibition and binds pirenzepine with low affinity; and the low affinity site or conformation, which increases [^{3}H]cyclic GMP levels and binds pirenzepine with high affinity. Partial agonists can apparently induce only one conformation of the receptor, the high affinity site or conformation, and thus are able to mediate only the reduction in [^{3}H]cyclic AMP levels.

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[1] The abbreviations used are: K_D or K_i, equilibrium binding dissociation constant; K_H equilibrium dissociation constant for the high affinity agonist site; K_L, equilibrium dissociation constant for the low affinity agonist site; B_H, capacity of the high affinity agonist binding site; B_L, capacity of the low affinity agonist binding site; EC_{50}, effective concentration for response at 50% of maximum; IBMX, 3-isobutyl-1-methylxanthine; NMS, *N*-methylscopolamine; PGE_1, prostaglandin E_1; PBS, phosphate-buffered saline solution; QNB, L(−)quinuclidinyl benzilate; R_H, the high affinity receptor; R_L, the low affinity receptor; V_{max}, maximal response.

MATERIALS AND METHODS

Cell culture. Clone N1E-115 cells were grown as described (13) in modified Dulbecco's minimal essential medium supplemented with 10% fetal or newborn bovine serum. Confluent cells were detached from flasks with Puck's D_1 solution, collected by low speed centrifugation, and washed in a physiological isosmolar PBS (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) containing sucrose. Low subculture cells were used to minimize variations in binding and response parameters due to subculturing (10).

Cyclic nucleotide assays. Detached and washed N1E-115 cells were suspended in 3 ml of PBS (approximately 2–5 × 10^6 cells/ml) and transferred to a 25-ml Erlenmeyer flask. The cells were labeled with 30 μCi of [^{3}H]adenine (Amersham, 22 Ci/mmol) or with 30 μCi of [^{3}H]guanine (Amersham, 7.2 Ci/mmol) in a shaking water bath at 37° for 45 min. The cells labeled with [^{3}H]adenine were assayed for agonist inhibition of PGE_1-stimulated [^{3}H]cyclic AMP formation as described (14). Briefly, after labeling, the cells were diluted with PBS at 37° to 500,000 cells/ml and distributed in 230-μl aliquots into the wells of a multiwell tray at 37° in the shaker bath. Then 40 μl of either PBS (control) or inhibitor (at the final concentration indicated) was added to each well along with 50 μl of IBMX (0.4 mM final concentration). Cells were then allowed to equilibrate for 30 min, after which time 80 μl of either PBS (basal), PGE_1 (control, 1 μM final concentration), or PGE_1 + agonist (1 μM PGE_1 final concentration, agonist at indicated concentration) was added. After a 10-min incubation (the time required for the effect of the agonist to reach a maximum), the reaction was stopped with 30 μl of 50% (w/v) trichloroacetic acid. The [^{3}H]cyclic AMP levels were then determined as described (8).

Cells labeled with [^{3}H]guanine were assayed for [^{3}H]cyclic GMP formation as described (13). Briefly, the cells were diluted with PBS at 37° to 300,000 cells/ml and distributed into the wells of a multiwell tray at 37° in the shaker bath in 320-μl aliquots. Then 40 μl of PBS (control) or inhibitor drug (final concentrations as indicated) was added followed by a 30-min (pirenzepine) or 5-min (partial agonist) equilibration. The presence of IBMX is not necessary to observe [^{3}H]cyclic GMP formation and had no effect on either carbachol EC_{50} values or pirenzepine K_D values, so it was omitted from this assay. After the equilibration, 40 μl of PBS (basal) or agonist (final concentrations as indicated) was added. The reaction was stopped at 30 sec with 30 μl of 50% trichloroacetic acid. [^{3}H]Cyclic GMP levels were then determined as described previously (13).

For the data of Table 3 and Fig. 5, all 10 agonists were assayed for one response in a given experiment. Sister cells were then incubated with the other radiolabeled base and the 10 agonists were assayed together in the other response. Similarly, for comparing EC_{50} values for the [^{3}H]cyclic AMP and the [^{3}H]cyclic GMP response to carbachol, some parallel incubations with separately labeled sister cells were performed (some of the data of Table 1 were generated this way). Preliminary experiments performed with nicotine and *d*-tubocurarine showed that the two cyclic nucleotide responses were not mediated by nicotinic receptors. These responses were completely inhibited by atropine, showing their muscarinic character (data not shown; see Refs. 13 and 14a).

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 unlabeled muscarinic drugs (Sigma Chemical Company, St. Louis, MO) were performed. Radioligands were obtained from New England Nuclear (Boston, MA). Binding experiments were generally performed with intact N1E-115 cells in suspension (150,000–300,000 cells/tube) in PBS. Each ligand concentration was assayed in triplicate in 2-ml total volume. For binding to intact cells, assay temperature was maintained at 15° to prevent desensitization by carbachol during the assay (9, 10, 15). Binding assays with homogenates were performed at 37°. Bound ^{3}H-labeled ligand after 75 or 45 min ([^{3}H]QNB in intact cells or homogenates, respectively) or after 45 or 30 min ([^{3}H]NMS in intact cells or homogenates, respectively) was separated from free ligand by vacuum filtration over Whatman GF/B filters (Whatman, England). ^{3}H-Ligands were at 90% or more of equilibrium within these times of incubation. The filters were then washed four times with 4 ml each of ice-cold 0.9% NaCl. Filters were solubilized for 2 hr in Safety-Solve (Research Products International, Elk Grove Village, IL) and radioactivity was measured by liquid scintillation counting. The maximal number of binding sites and equilibrium dissociation constant for [^{3}H]QNB were determined by Scatchard analysis (16) of the saturation [^{3}H]QNB binding. [^{3}H]QNB binding was monophasic and the dissociation constant was 0.38 ± 0.02 nM (n = 13) at 15° for intact cells and was 0.05 ± 0.01 nM (n = 9) at 37° for homogenates and did not vary with subculture. The equilibrium dissociation constant for [^{3}H]NMS, obtained by saturation binding, was 0.21 ± 0.01 nM (n = 17) at 15° for intact cells and was 0.31 ± 0.04 nM (n = 3) at 37° for homogenates; [^{3}H]NMS binding also was monophasic and this constant did not vary with subculture. The ^{3}H-antagonist dissociation constant was fixed as a constant in the independent one-site or two-site (as appropriate) ^{3}H-antagonist-agonist competition model used in computer analysis of agonist-binding sites. This iterative nonlinear analysis [described in detail in McKinney and Coyle (17)] provides estimates of the equilibrium dissociation constants (K_H, K_L,) for carbachol or another full agonist bound to the high affinity and low affinity agonist sites and the capacities (B_H and B_L) for these two sites. Data for partial agonists (actually behaving as antagonists in the [^{3}H]cyclic GMP response) generally were fit to a single-site model. Equilibrium binding with pirenzepine was also performed by competition with [^{3}H]QNB or [^{3}H]NMS (with intact cells at 37°) and the determination of parameters for two sites was accomplished similarly. The concentration of [^{3}H]QNB or [^{3}H]NMS used in competition assays was in the range of 0.2–0.6 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 or 10 μM NMS was usually used. One μM atropine occasionally revealed about 10–15% more [^{3}H]QNB "sites." Therefore, the asymptotic value of [^{3}H]QNB bound at 10 mM carbachol or binding in the presence of 10 μM NMS 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.

There was usually less specific muscarinic 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 and in cells sonicated just before filtration (to allow washout of the radioligand taken up by cells). Calculated maximal specific binding was the same in both cases (160 ± 10 and 152 ± 8 fmol/10^6 cells, respectively) and indicated that the binding assay with intact cells reflected true specific receptor binding. That specific binding was less in homogenates of these cells indicated an instability of the muscarinic receptor in homogenates. Though we usually performed binding to muscarinic receptors in intact cells at 15°, in some experiments (Table 4), binding in homogenates at 37° was performed to show that temperature did not largely affect the binding constants (also see Ref. 10). Our data are consistent with the concept that the bulk of the binding sites are located on the surface of the cell, and thus are accessible to muscarinic ligands in the medium. We further examined this postulate in several experiments by analyzing the competition of [^{3}H]NMS (an impermeant radioligand) and carbachol; the capacities and equilibrium dissociation constants for the high and low affinity agonist sites obtained in this manner were essentially identical (n = 5) to those obtained by [^{3}H]QNB/carbachol binding (data not shown). Therefore, [^{3}H]NMS/carbachol data were combined with [^{3}H]QNB/carbachol data. Additionally, when intact cells were incubated with [^{3}H]QNB and with various concentrations of carbachol and then filtered and washed with water (to lyse the cells), the K_H (0.7 μM) and K_L (17 μM) for carbachol were the same as for cells filtered intact. Generally, at the concentrations of [^{3}H]QNB and [^{3}H]NMS normally used with intact cells (0.2 and 0.6 nM, respectively), the level of nonspecific binding was about 15–30% and 5–10% of the total binding, respectively. Generally, 24 pairs of data (free concentration of competing agent and amount of [^{3}H]QNB or [^{3}H]NMS specifically 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.

Estimated limits to the binding assay and computer analysis. The limits of sensitivity of the radioligand competitive binding assay for the detection of multiple sites was roughly estimated as follows. For an experiment with [^{3}H]NMS in competition with a partial agonist (arecoline), the program was observed to give two binding sites (52:48) with a sum of squares of 73.4 and a root mean square error of 2.0. A one-site fit gave a sum of squares of 77.6 and a root mean square error of 2.1. The two-site fit was not significantly better (F = 0.57, $p > 0.1$) and one class of sites was deemed present for arecoline. However, the K_H and K_L values in the two-site fit were 4.3 and 11 μM. Thus, with nearly equal proportions of sites and for 24 data points, each with typical error (about 3% of the bound value), the experiment probably will not "detect" two sites if their binding dissociation constants do not differ by more than 3- or 4-fold. Routinely in binding with intact cells (where error is 2-fold lower than with homogenates), there were significantly better fits to two sites when the ratios of binding constants was as low as 10. The lowest proportion of a second class of binding sites that can be reliably detected with this type of computer analysis is about 10% (18). In several experiments (see Table 4), a small proportion (4–13%) of a second site was detected and the F ratio values were large enough for significance, but because the finding did not repeat and because the proportion was low, the second (minor) site was not considered significant and only the major site is reported.

This survey indicates that there are limits to the use of the analytical technique in confirming whether multiple sites are present. Generally, the K_D ratios must be equal to or larger than about 10 (a conservative estimate) and/or the proportion of the minor site must exceed about 10%.

Other techniques for analyses of data and statistical tests. The concentration-response curves for agonists were analyzed by a Hewlett-Packard 9845B computer using programs that fit the data directly with a logistic function (19), minimizing the sum of squares and providing the EC_{50} value, the maximal response, and the equivalent of the Hill slope. Though responses sometimes gave Hill slopes not equal to unity, these data were not analyzed with multicomponent models, because steep or shallow Hill slopes in responses can arise from nonlinear mechanisms not involving receptor self-interactions. It has been assumed herein that a given response arises from noninteracting receptors. For the analysis of competitive antagonism by antagonists or partial agonists, where the concentration-response curves were parallel and there was no depression of the V_{max}, a computer program provided the K_D value of the inhibitor calculated from the dose-ratio values. Additionally, they were plotted on a linear plot versus the inhibitor concentration and the line was constrained to pass through the origin; the inverse of the slope is the inhibitor equilibrium dissociation constant (20). This type of plot assumes competitive interactions and generally gives more accurate statistical treatment to the data than the Schild plot (20). The results of such analyses are shown in plot form

in Figs. 1, 3, and 8. However, the slopes from conventional Schild plots (21) are also given where appropriate.

For data reported as arithmetic means (± standard errors) of data obtained in individual experiments, the statistical test used was the unpaired *t* test (two-tailed). Parameter values reported as significant were for $p < 0.05$. The computer program used for binding analysis (18) also provides an estimate of the standard error of parameter values. For testing the significance of fit to binding models, the *F* ratio method was used (19).

RESULTS

Stimulation of the cyclic nucleotide responses by carbachol and inhibition by pirenzepine. Carbachol stimulates [^{3}H]cyclic GMP formation in N1E-115 cells with an EC_{50} in the range of 18–110 μM and inhibits PGE_1-mediated [^{3}H]cyclic AMP increases in these cells with an EC_{50} in the range of 1–4 μM, depending on the subculture (10). The ratio of the two EC_{50} values is generally about 25 or greater. In nine experiments, the EC_{50} values for the two responses were determined in parallel assays with sister cells (see Materials and Methods) and shown to differ by this factor. Table 1 shows typical EC_{50} values for carbachol's mediation of the two responses. These EC_{50} values are significantly different ($p < 0.001$). The relatively large standard errors arise from taking the average of the EC_{50} values over several subcultures (see Ref. 10).

In the experiment shown in Fig. 1A, the control concentration-response curve for the stimulation of [^{3}H] cyclic GMP by carbachol had an EC_{50} of 18 μM. This response curve was shifted progressively to the right by 5 and 7.5 nM pirenzepine. At these concentrations, pirenzepine did not depress the maximal response, indicating a competitive type of antagonism. The modified Schild plot (20) shown in Fig. 1B for 10 such experiments gave a K_D of 6.5 nM for the binding of pirenzepine at the site at which carbachol mediates [^{3}H]cyclic GMP formation. When the curves were analyzed by the method of Schild (21), the slope was −1, which is the theoretical value for competitive inhibition.

Increasing the concentration of pirenzepine above the level at which it shifted the [^{3}H]cyclic GMP response curve about 2.5-fold resulted in depression of the maximal response (Fig. 2A). This behavior may be accounted for by postulating the existence of a slowly dissociating pirenzepine-receptor complex, at which carbachol in a 30-sec assay is unable to bind competitively. We have found that other muscarinic antagonists can display the same behavior (data not shown). Alternatively, it may be explained by a noncompetitive effect of pirenzepine on muscarinic receptors. The double reciprocal plot of the curves (Fig. 2B) presented a mixed competitive/noncompetitive type of antagonism. The secondary plot (slope versus [*I*]), shown in the inset to Fig. 2B, gave a

TABLE 1

Equilibrium dissociation constants and EC_{50} values for carbachol

Murine neuroblastoma clone N1E-115 cells were assayed for the EC_{50} values for carbachol in the stimulation of [^{3}H]cyclic GMP and inhibition of PGE_1-cyclic AMP production as described in Materials and Methods. The equilibrium dissociation constants for carbachol were determined in intact N1E-115 cells using radioligand binding with [^{3}H]methscopolamine chloride as described in Materials and Methods. Binding and response data are averaged over the same early subcultures (9 and 10). The values shown are the arithmetic mean ± standard error.

Assay	EC_{50}[a]	K_L[b]	K_H[b]
	μM	μM	μM
Radioligand binding with intact cells ($n = 14$)		29 ± 6 (63%)	1.2 ± 0.2 (37%)
[^{3}H]Cyclic GMP stimulation ($n = 14$)	37 ± 8		
PGE_1-[^{3}H]cyclic AMP inhibition ($n = 14$)	1.5 ± 0.2		

[a] The concentration required to produce 50% of the maximal response (either [^{3}H]cyclic GMP synthesis or [^{3}H]cyclic AMP inhibition).

[b] Values are the equilibrium dissociation constants for carbachol versus [^{3}H]methscopolamine. Subscripts *L* and *H* represent the low and high affinity carbachol sites, respectively, as referred to in the text. The relative proportions of the two sites are shown in parentheses.

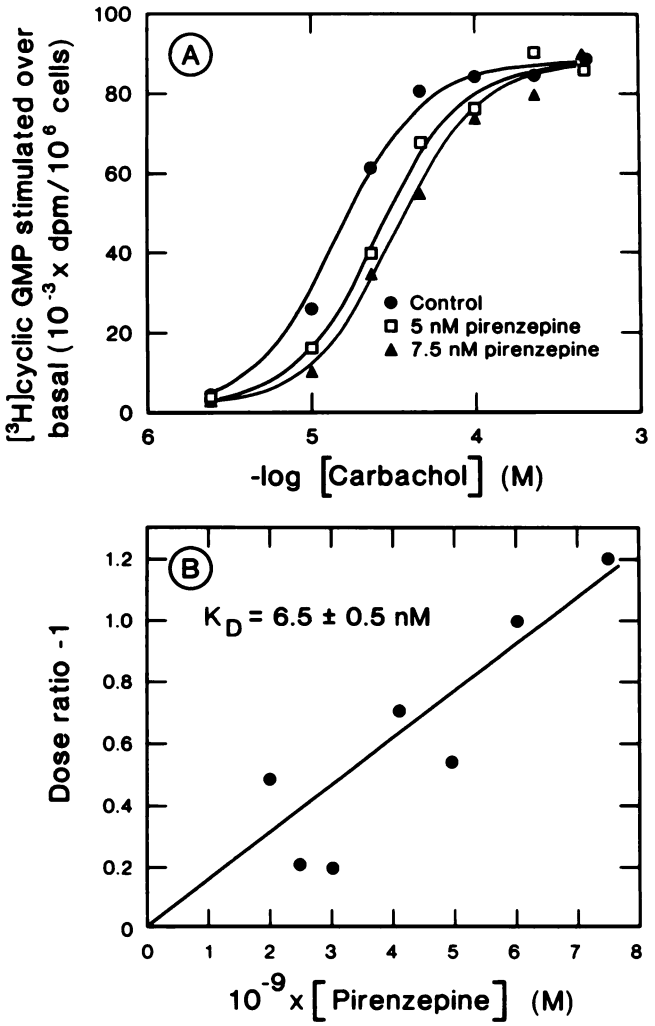

FIG. 1. *Effect of low concentrations of pirenzepine on the concentration-response curve for carbachol in elevating [^{3}H]cyclic GMP levels in N1E-115 cells*

A, concentration-response curves for control (●), in the presence of 5 nM pirenzepine (□), and in the presence of 7.5 nM pirenzepine (▲). B, modified Schild plots of several inhibition experiments with pirenzepine. The slope of the line (K_D) and its standard error were obtained after linear regression. The K_D for pirenzepine for inhibiting the [^{3}H] cyclic GMP response to carbachol was 6.5 ± 0.5 nM. Cells were subcultures 9–11.

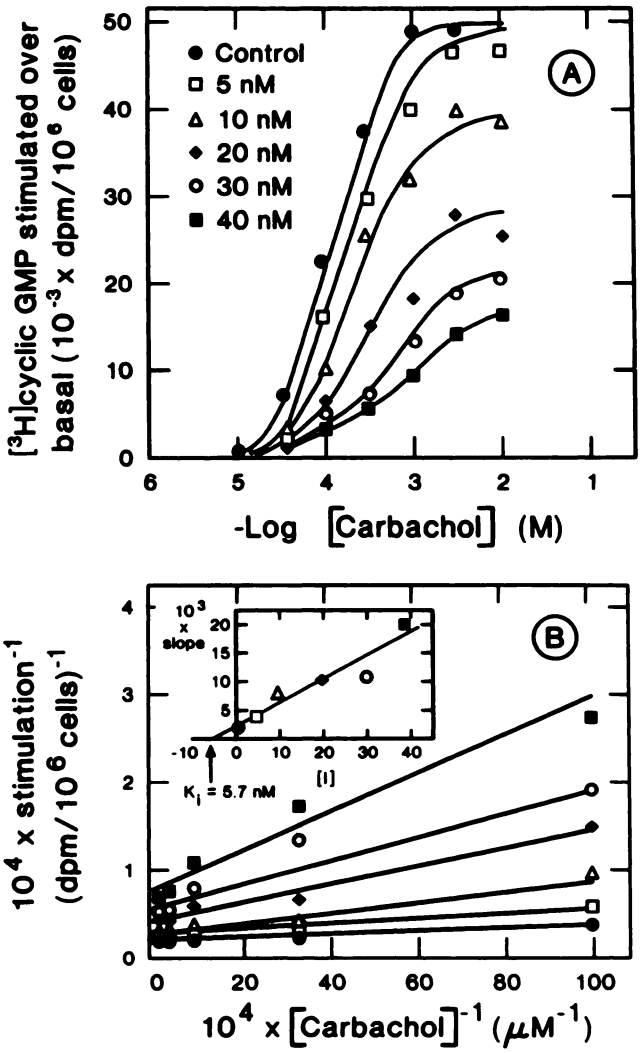

FIG. 2. *Effect of higher concentrations of pirenzepine on the concentration-response curve for carbachol in elevating [³H]cyclic GMP levels in N1E-115 cells*

At least four experiments demonstrated this effect. A, depression of the V_{max} with pirenzepine. Various concentrations of pirenzepine (5 nM, □; 10 nM, △; 20 nM, ◆; 30 nM, ○; and 40 nM, ■) were incubated with prelabeled N1E-115 cells for 30 min prior to assay with a range of carbachol concentrations. B, double reciprocal plot of the data in A. Inset is a secondary plot of the slopes of the lines versus pirenzepine concentration. The x intercept equals $-K_i$ (−5.7 nM). Cells were subculture 11.

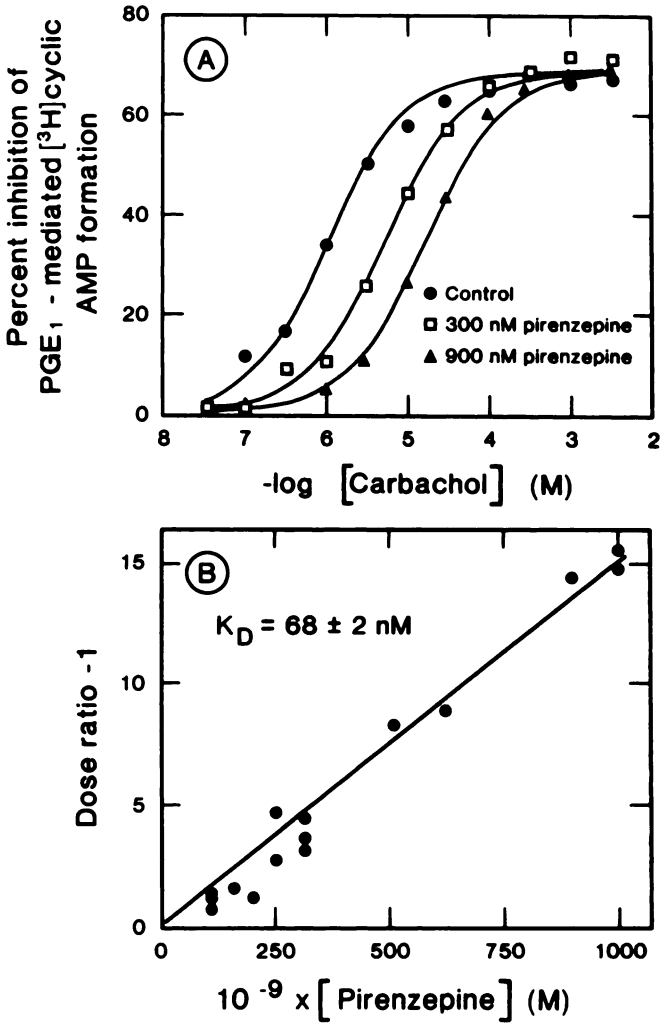

FIG. 3. *Effect of pirenzepine on the concentration-response curve for carbachol in the reduction of PGE_1 (1 μM)*-mediated increases in [³H] cyclic AMP levels in prelabeled N1E-115 cells

A, parallel shift of the concentration-response curve for carbachol by 300 nM (□) and 900 nM (▲) pirenzepine. B, composite modified Schild plot of 17 inhibition experiments with pirenzepine. The slope of the line (K_D) and its standard error were obtained after linear regression. The K_D value for pirenzepine for inhibiting the [³H]cyclic AMP reduction by carbachol was 68 ± 2 nM.

calculated K_i for pirenzepine of 5.7 nM. (The average of four such experiments was 4.2 ± 0.7 nM.) This value is not significantly different from that obtained by the initial competitive effect (6.5 nM) and may indicate a common site of action for the two stages of antagonism.

Much higher concentrations of pirenzepine were needed to effect a shift in the other cyclic nucleotide response to carbachol (Fig. 3A). The control curve in this experiment had an EC_{50} value for carbachol of 1 μM. Pirenzepine at 300 and 900 nM caused parallel shifts to the right in the response curve of 5- and 16-fold, respectively. Even with such a shift, pirenzepine never depressed the maximal response at this receptor. This was an indication that pirenzepine was acting in competitive fashion. The data shown in Fig. 3A were simultaneously fit by computer to a model assuming competitive action of pirenzepine (19) and 11 experiments were performed in which 17 sets of control curves and curves in the presence of the antagonist were obtained. The average value for pirenzepine's dissociation constant using this competition model was 93 ± 8 nM (range, 61–189 nM). When the data from all the experiments were analyzed as a composite by the method of Schild (21), the slope was −1.28 ± 0.03, which was significantly different from the −1 value usually taken to indicate competitive antagonism. The meaning of the differing slope is not understood, but could arise from a number of nonspecific effects and might not necessarily mean that a model other than one of competition should be assumed. The modified Schild plot (Fig. 3B) which constrains the data to a competition model yielded a K_D of 68 nM for these data, a value differing by a factor of more than 10 from pirenzepine's effect on [³H]cyclic GMP formation.

Binding studies with carbachol and pirenzepine. The response/inhibition data with pirenzepine indicated that different subtypes of receptors might be involved in the two cyclic nucleotide responses to carbachol. We substantiated this finding with binding data. Carbachol binds to two major muscarinic receptor subtypes in intact N1E-115 cells (10). A typical competition curve for [^{3}H]NMS-carbachol in intact cells at 15° is shown in Fig. 4. The data do not lie on a monophasic curve (dotted line), but are adequately fit with an independent two-site model (solid line) giving $K_H = 1.9$ μM and $K_L = 26.8$ μM, with 60% of the sites having the higher affinity. This proportion of sites varies depending on subculture as previously described (10). In Table 1, the proportion of high affinity agonist sites, averaged in several experiments with early subcultures, was 37 ± 4% ($n = 14$) but this actually varied from 32–85%, depending on the particular subculture. With either intact cells at 15° (Table 1) or homogenates at 37° (see Ref. 10), the two major sites present for the binding of carbachol (R_H, R_L) had equilibrium dissociation constants (K_H, K_L) that were remarkably similar to the EC_{50} values for the two responses.

[^{3}H]QNB-pirenzepine or [^{3}H]NMS-pirenzepine competition binding experiments also gave biphasic curves (an example is shown in Fig. 4). Fitting the data with a two-site binding model gave binding constants for pirenzepine of 11 and 190 nM (Table 2). These values were similar to, but somewhat higher than, the two values for pirenzepine obtained by the dose-ratio method in the above inhibition experiments. The ratio of pirenzepine's K_D values in the binding experiments was 14.9 ± 1.5 ($n = 6$). In four of the 11 inhibition experiments, the K_D values for the inhibition of each response by pirenzepine, determined with sister cells in the same experiment, were 10 ± 2 and 133 ± 21 nM for the [^{3}H]cyclic GMP and [^{3}H]cyclic AMP responses, respectively, differing by a factor of 14.3 ± 1.5. The K_D values for pirenzepine's inhibition of the two responses were significantly different ($p < 0.01$). By either type of assay (binding or dose inhibition), two muscarinic sites for pirenzepine were evidently present in N1E-115 cells, with K_D values of about 6–11 nM for the high affinity constant and 90–190 nM for the low affinity constant. The average proportion of the high affinity pirenzepine sites was 28 ± 5% ($n = 6$) and varied from assay to assay (14–46%).

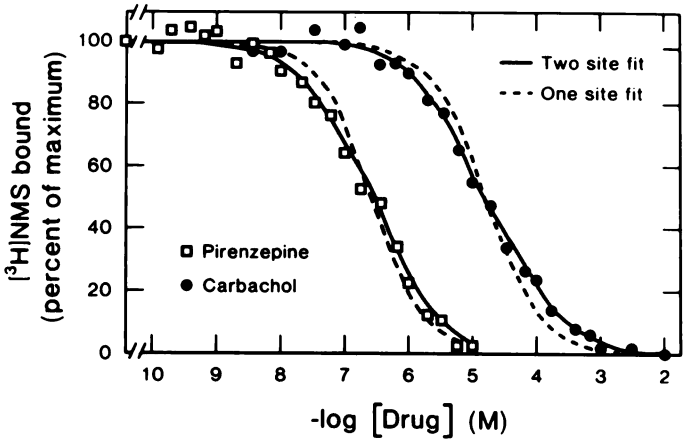

FIG. 4. *Competition binding with [^{3}H]NMS and pirenzepine (□) and carbachol (●)*

The binding assay was performed with intact N1E-115 cells at 15° (for carbachol) or 37° (for pirenzepine), as described in Materials and Methods. The smooth curve is the computer-generated best fit to a two-site model; the broken line is the calculated binding curve for the muscarinic ligands competing at a single class of receptors. For both ligands, the two-site model fit significantly better (by the partial F test; $p < 0.001$). Cells were subcultures 9 and 10.

TABLE 2

Equilibrium dissociation constants for pirenzepine determined by binding or response inhibition experiments

The equilibrium dissociation constants for pirenzepine in N1E-115 cells were determined for intact cells of several early subcultures (8–11) either in competition experiments between pirenzepine and [^{3}H]QNB (see Materials and Methods) or by using the dose-ratio method for the competitive inhibition of the carbachol mediated-responses of [^{3}H]cyclic GMP stimulation or inhibition of PGE_1-[^{3}H]cyclic AMP formation. In the four experiments shown for the dose-ratio method, the two constants were determined by parallel assays of sister cells. The values are the averages ± standard error.

Method of determination	K_D[a]	High affinity binding constant[b]	Low affinity binding constant[b]
	nM	*nM*	*nM*
Radioligand binding with intact cells (37°, $n = 6$)		11 ± 3 (28%)	190 ± 20 (72%)
Dose-ratio from [^{3}H] cyclic GMP assays ($n = 4$)	10 ± 2		
Dose-ratio from PGE_1-[^{3}H]cyclic AMP assays ($n = 4$)	133 ± 21		

[a] Equilibrium dissociation constant for the inhibitor obtained by using the dose-ratio method.

[b] Values are the equilibrium binding dissociation constants for pirenzepine versus [^{3}H]QNB. Relative proportions of sites are shown in parentheses.

Mediation of the two cyclic nucleotide responses by different agonists. The two cyclic nucleotide responses in N1E-115 cells were differentially mediated by various agonists. Some agonists were able to stimulate the [^{3}H]cyclic AMP response but unable to stimulate the [^{3}H]cyclic GMP response. The efficacies and potencies of 10 agonists in the stimulation of these responses are shown in Table 3. Typical experiments are shown in Fig. 5. Acetylcholine was the most potent and effective at each response. Six other agonists (carbachol, *cis*-dioxolane, arecoline, oxotremorine, muscarine, and methacholine) were as effective in reducing [^{3}H]cyclic AMP levels as acetylcholine (thus, their order of ranking by efficacy was probably not significant); the other three drugs (bethanecol, McN-A343, and pilocarpine) were partial agonists at this response. In the [^{3}H]cyclic GMP response, by contrast, there were only two agonists (carbachol and methacholine) that were as effective as acetylcholine; the other seven were partially or completely unable to increase [^{3}H]cyclic GMP levels. The [^{3}H]cyclic GMP response to oxotremorine (shown in parentheses in Table 3) is likely to be nonspecific as this agonist at

TABLE 3

Rank ordering of 10 muscarinic agonists for their effects on cyclic nucleotide levels in N1E-115 cells

Assays (n = 5 to 8 for each agonist in each assay) for the two responses were performed in parallel with sister cells (subcultures 8–11) as described in the legend to Fig. 5 and in Materials and Methods.

Inhibition of PGE_1-mediated [^{3}H]cyclic AMP stimulation		Stimulation of the level of [^{3}H]cyclic GMP	
Agonist	Efficacy	Agonist	Efficacy
	%		%
A. Ranking by efficacy			
Acetylcholine	100	Acetylcholine	100
Carbachol	103 ± 2	Carbachol	99 ± 2
cis-Dioxolane	96 ± 2	Methacholine	96 ± 3
Arecoline	96 ± 5	*cis*-Dioxolane	83 ± 3
Oxotremorine	95 ± 2	Muscarine	69 ± 4
Muscarine	95 ± 5	Oxotremorine[a]	(64 ± 6)
Methacholine	93 ± 2	Bethanecol	18 ± 2
Bethanecol	70 ± 7	Arecoline	6 ± 1
McN-A343	50 ± 5	Pilocarpine	2 ± 1
Pilocarpine	42 ± 5	McN-A-343	2 ± 1
Agonist	EC_{50}	Agonist	EC_{50}
	μM		μM
B. Ranking by potency			
Acetylcholine	0.4 ± 0.1	Acetylcholine	23 ± 3
Oxotremorine	0.4 ± 0.1	*cis*-Dioxolane	60 ± 20
cis-Dioxolane	1.6 ± 0.3	Muscarine	90 ± 20
Arecoline	1.8 ± 0.2	Methacholine	110 ± 10
Carbachol	1.9 ± 0.3	Carbachol	110 ± 20
Methacholine	2.1 ± 0.6	Arecoline	160 ± 40
Muscarine	2.1 ± 0.6	Bethanecol	1300 ± 200
Pilocarpine	2.8 ± 0.8	Oxotremorine[a]	(2800 ± 200)
McN-A343	4 ± 1	Pilocarpine	NS[b]
Bethanecol	80 ± 10	McN-A343	NS

[a] Oxotremorine at millimolar levels damaged cellular integrity.
[b] NS, no stimulation.

millimolar levels (a concentration more than 1000 times its binding constant) visibly disrupted cellular integrity. This was not observed with the other drugs, even at millimolar levels. In N1E-115 cells, the putative M_1 agonist McN-A343 had little effect on [^{3}H]cyclic GMP levels.

Of the 10 agonists, the four that were essentially unable to mediate [^{3}H]cyclic GMP increases were oxotremorine, arecoline, pilocarpine, and McN-A343. A fifth agonist, bethanecol, did significantly increase [^{3}H]cyclic GMP, but only about 18% as well as acetylcholine. Though Fig. 5A may seem to indicate that a plateau was not reached at 3 mM for bethanecol, the four other experiments did show this. The lack of effect of these agonists was not due to a different time of maximum [^{3}H]cyclic GMP formation, since bethanecol, oxotremorine, pilocarpine, arecoline, and McN-A343 caused peak [^{3}H]cyclic GMP responses at 30 sec in assays that sampled effects of agonists at up to 4 min (data not shown). The other five agonists were able to mediate [^{3}H]cyclic GMP formation, but two (*cis*-dioxolane and muscarine) were not as effective as acetylcholine. The three agonists that could mediate both responses 100% were acetylcholine, methacholine, and carbachol; these are referred to as "full" agonists.

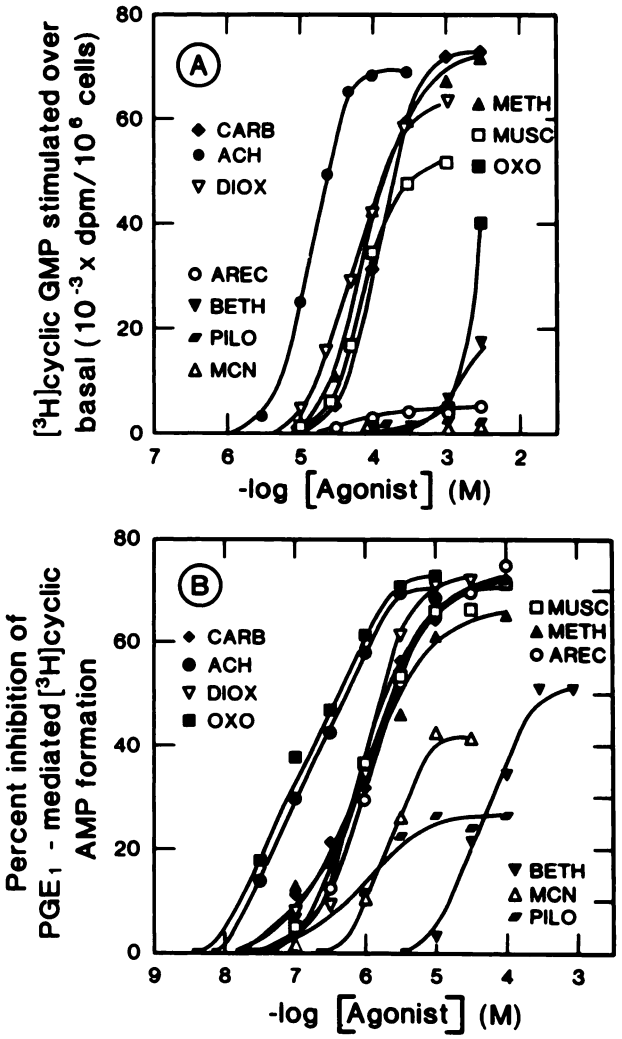

FIG. 5. *Concentration-response curves for 10 agonists in the two cyclic nucleotide responses in N1E-115 cells*

A, curves for the muscarinic receptor-mediated increase in [^{3}H] cyclic GMP levels after prelabeling with [^{3}H]guanine. B, curves for the muscarinic receptor-mediated inhibition of PGE_1 (1 μM)-mediated increases in [^{3}H]cyclic AMP levels after prelabeling with [^{3}H]adenine. The experiments in A and B were performed in parallel with all 10 agonists in the same experiment. Each experiment was repeated at least five times. *METH*, methacholine; *MUSC*, muscarine; *OXO*, oxotremorine; *CARB*, carbachol; *ACH*, acetylcholine; *DIOX*, *cis*-dioxolane; *AREC*, arecoline; *BETH*, bethanecol; *PILO*, pilocarpine; *MCN*, McN-A343. Cells were subcultures 8–12.

Several of the agonists able to mediate at least some response in both assays had very similar EC_{50} values for the [^{3}H]cyclic AMP response (1.6–2.8 μM) and also had very similar EC_{50} values for the [^{3}H]cyclic GMP response (60–110 μM) (Table 3B). Thus, the ranking by potency for these agonists does not necessarily support differing structure-activity relationships for the two responses. However, it should be noted that each agonist that could mediate the [^{3}H]cyclic GMP response always did so with a higher EC_{50} value than that for its effect on [^{3}H]cyclic AMP levels.

There was no simple correlation between efficacy and

potency for these drugs when considered in a particular response. However, for bethanecol, *cis*-dioxolane, muscarine, methacholine, acetylcholine, and carbachol (agonists that were able to mediate both responses significantly), there was a correlation between their efficacy and the ratio of their EC_{50} values (EC_{50} at [^{3}H]cyclic GMP response/EC_{50} at [^{3}H]cyclic AMP response) (Fig. 6). For these six agonists, which mediated 70–103% of the [^{3}H]cyclic AMP response, the correlation coefficient was 0.92 and the dependence on the ratio was slight. For these six agonists, which mediated 18–100% of the maximal [^{3}H]cyclic GMP response, the correlation coefficient was 0.96 and the dependence was much more pronounced (Fig. 6). These correlations are interesting because of the recent prediction that the efficacy of a muscarinic agonist would depend on the ratio of their high and low affinity binding constants (22). Though here we have used EC_{50} values, these may in fact be identical or directly related to their binding constants.

Binding studies with agonists unable to stimulate [^{3}H] cyclic GMP synthesis. In the above experiments, carbachol bound to two sites and stimulated as well as acetylcholine the two responses which were inhibited by pirenzepine in a way indicating action at two sites. It was of interest to determine if partial agonists (partial in the sense that they mediated only one response) bound to one or two sites. Binding to only one site would suggest that only one agonist-receptor conformation would be appropriate for eliciting a response, and this would be the conformation mediating [^{3}H]cyclic AMP reduction.

Typical competition binding curves for arecoline, bethanecol, oxotremorine, and pilocarpine appear in Fig. 7 with that of carbachol, which was included as an internal control. It is readily apparent, and it was confirmed by statistical analysis, that binding with the four partial

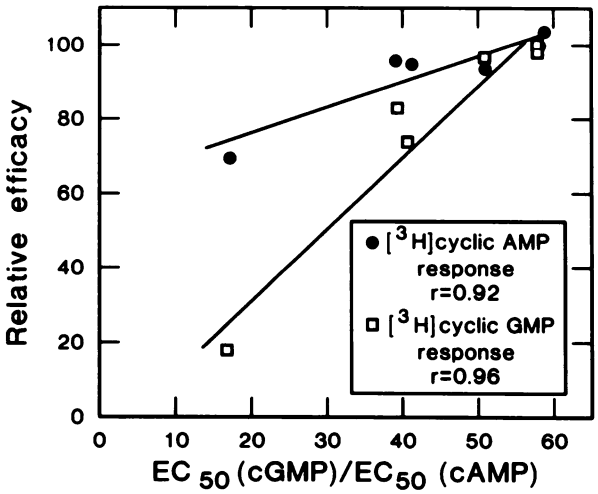

FIG. 6. *Correlation between relative efficacy and the potency ratio for the six muscarinic agonists that significantly stimulated both cyclic nucleotide responses (excluding oxotremorine)*

Efficacy is expressed as percentage of the maximal response to acetylcholine. The potency ratio is the number obtained by dividing a given agonist's EC_{50} value for elevating [^{3}H]cyclic GMP levels by its EC_{50} value for reducing [^{3}H]cyclic AMP levels. The six agonists were acetylcholine, bethanecol, carbachol, *cis*-dioxolane, methacholine, and muscarine. The data for the plot were derived from Table 3.

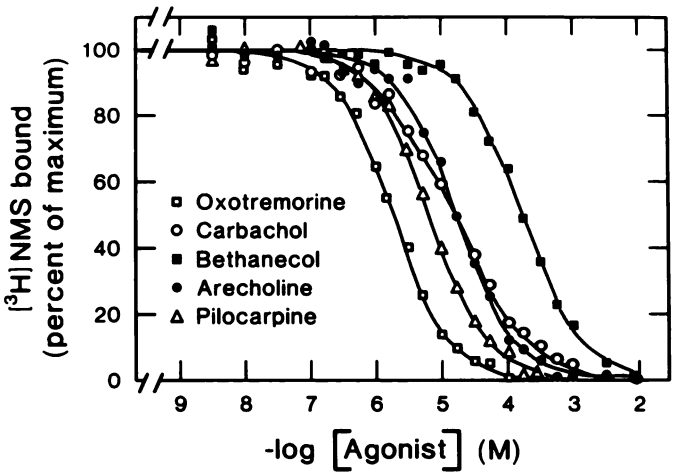

FIG. 7. *Competition binding with [^{3}H]NMS and four partial agonists*

Arecoline, bethanecol, oxotremorine, and pilocarpine binding were compared with that of carbachol in the same assay. Each curve was comprised of 24 points, each determined in triplicate. The smooth line indicates the computer-generated best fit to a one-site model (for the partial agonists) or a two-site model (for the full agonist). The experiment shown was performed with intact N1E-115 cells at 15° and was repeated three times. Three other experiments were performed with homogenates at 37°. The computer-derived binding constants are shown in Table 4. Cells were subcultures 9–12.

agonists was monophasic, while the binding curve for the full agonist was flatter, reflecting distribution of the receptor-agonist complexes between two states. Since [^{3}H]NMS binds to all the receptors with a single binding constant, since in these assays the [^{3}H]NMS concentration was sufficient to occupy >75% of the binding sites, and since all the agonists compete for all the specific [^{3}H]NMS binding, it must be concluded that the partial agonists, like carbachol, bind to all the receptors, and not to a subset; but unlike carbachol, they bind monophasically, insofar as the method can detect (see Materials and Methods). This experiment was performed three times at 15° with intact cells and three times at 37° in homogenates (see Table 4). At both temperatures, the partial agonists bound monophasically; their K_D values at the two temperatures were similar, differing by being only 1.1- to 2.9-fold higher (average, 2.1-fold) at 37°. Interestingly, as shown in the table, the binding constants for these agonists were very similar to their respective EC_{50} values for reducing [^{3}H]cyclic AMP levels; the only one with significantly different values was arecoline ($p < 0.05$) (Tables 3B and 4).

This result with the binding of partial agonists suggested that they bound to the same receptors at which the full agonist bound and induced the [^{3}H]cyclic GMP response. Another way to show this is by inhibiting this response to the full agonist with the partial agonist. These experiments were performed by testing several concentrations of the partial agonists for their effect on the concentration-response curve for carbachol. All five of the partial agonists (arecoline, bethanecol, McN-A343, oxotremorine, and pilocarpine) behaved as competitive antagonists in the carbachol-[^{3}H]cyclic GMP response assay; that is, the concentration-response curve for carbachol was shifted in parallel to the right with increasing

TABLE 4

Binding parameters for four partial agonists determined at two temperatures

Binding experiments were performed at 15 and at 37° with four partial agonists and carbachol assayed together in a given experiment. Equilibrium dissociation constants for the partial agonists were also determined by the dose-ratio method in competition with the carbachol-[^{3}H]cyclic GMP response. Cells were subcultures 9–12.

Partial agonist	Binding constant of major site[a]		Equilibrium dissociation constant by dose-ratio
	15° (intact)	37° (homogenate)	
	μM		*μM*
Arecoline	4.5 ± 1 (3)	13 ± 2 (3)	5.2 ± 0.3 (7)
Bethanecol	70 ± 10 (3)	76 ± 7 (3)	110 ± 10 (5)
Oxotremorine	0.6 ± 0.1 (3)	1.7 ± 0.3 (3)	0.33 ± 0.03 (3)
Pilocarpine	2.3 ± 0.3 (3)	3.4 ± 0.2 (3)	7.3 ± 0.4 (5)
McN-A343	ND[b]	ND	7 ± 1 (5)

[a] In one (of six) experiments with bethanecol, there was a minor proportion (13%) of a second site; and in two (of six) experiments with pilocarpine there were minor proportions (4 and 11%). Though the fit to two sites was significant ($p < 0.05$), the low levels and inconsistent appearance of the second site was judged to be inconsequential.

[b] ND, not determined.

concentrations of the partial agonist, without a reduction of the maximal response for carbachol. One example (pilocarpine) is shown (Fig. 8). A computer program assuming competitive interactions provided the K_D values for these partial agonists, measured at the site at which carbachol mediates the [^{3}H]cyclic GMP response (Fig. 8B and Table 4). The Schild slopes for these drugs were −1.18 ± 0.13 (n = 5), not significantly different from −1, indicating competitive antagonism. For all the partial agonists, the K_D value obtained by dose-ratio (Table 4) agreed well with the respective EC_{50} value for the agonist in mediating the [^{3}H]cyclic AMP response (Table 3B). Their K_D values by dose-ratio were therefore also quite similar to their respective K_D values determined by binding assays at 15° in intact cells, and not much different from the K_D values obtained by binding at 37° in homogenates (Table 4). Oxotremorine was the exception, having a direct binding constant at 37° of 1.7 μM. Neither oxotremorine's K_d at 15° by binding nor its K_d at 37° by dose-ratio was significantly different from oxotremorine's EC_{50} value for reducing [^{3}H]cyclic AMP levels.

To support further the postulate that full agonists were able to differentiate between two muscarinic sites in N1E-115 cells, the binding of acetylcholine and methacholine was performed. These two agonists and carbachol were all fully able to stimulate both cyclic nucleotide responses (Table 3A). Several binding experiments with these three agonists were performed in parallel, with 100 μM physostigmine present to inhibit acetylcholinesterase. As shown in Table 5, acetylcholine and methacholine, like carbachol, bound to two sites in N1E-115 cells. The relative proportion of these two sites were

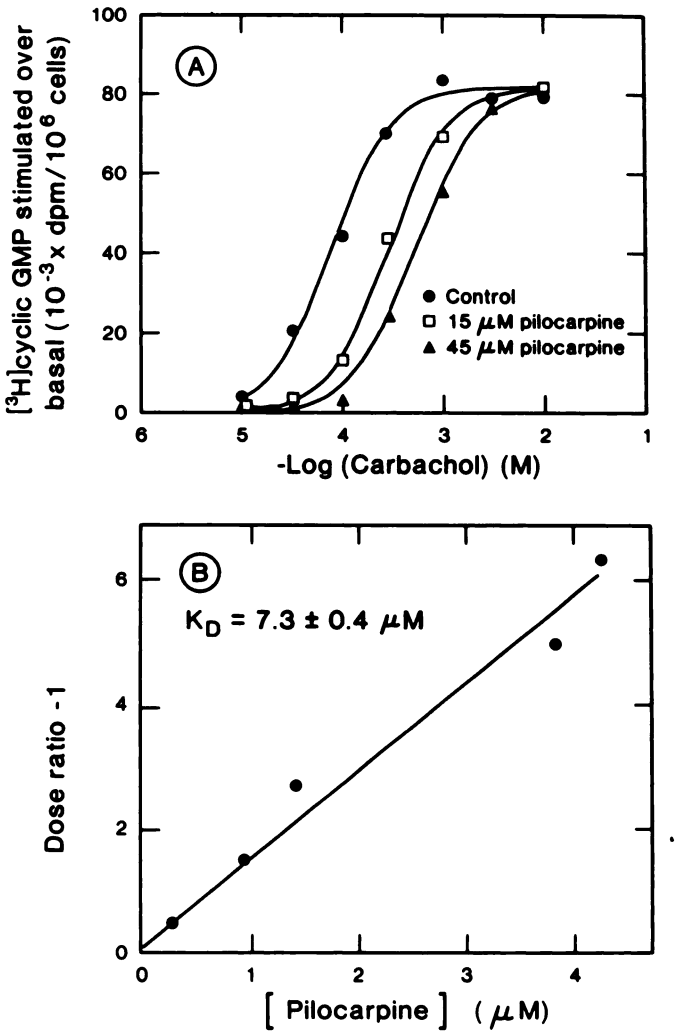

FIG. 8. *Effect of a partial agonist on the concentration-response of a full agonist in the [^{3}H]cyclic GMP response.*

The partial agonist pilocarpine was incubated with prelabeled N1E-115 cells for 5 min before challenge with various concentrations of carbachol. A, parallel shift of the concentration-response by 15 μM (□) and 45 μM (▲) pilocarpine. B, modified Schild plot of several inhibition experiments with pilocarpine. The derived K_D value for pilocarpine was 7.3 ± 0.4 μM.

quite similar for all three agonists, though varying somewhat from experiment to experiment, as shown in the table. Methacholine displayed a tendency to reveal or induce a higher proportion of low affinity sites.

DISCUSSION

By several pharmacologic techniques, we studied the heterogeneity of muscarinic receptor responses and binding sites in a homogeneous culture of neural cells (clone N1E-115). Full agonists (e.g., carbachol) mediated changes in intracellular levels of two cyclic nucleotides with very different EC_{50} values for each effect. The equilibrium binding experiments indicated that multiple sites for carbachol or other full agonists were present, with differing affinities for the agonist. Partial agonists appeared to bind to a single class of sites and they mediated only one response, i.e., [^{3}H]cyclic AMP inhibition. Each equilibrium dissociation constant for the

TABLE 5

Binding parameters for three full agonists

100 μM physostigmine sulfate was present in the incubations to prevent degradation of acetylcholine and methacholine by acetylcholinesterase. This drug did not have any significant effect on the binding of carbachol ($n = 3$, $p > 0.1$). These three agonists were fully able (relative to acetylcholine) to reduce [^{3}H]cyclic AMP levels or elevate [^{3}H]cyclic GMP levels in N1E-115 cells. Numbers in parentheses indicate number of experiments performed.

Full agonist	15° (intact cells)		Per cent B_L in three parallel experiments		
	K_H	K_L	1	2	3
	μM			%	
Carbachol	1.9 ± 0.5 (21)	39 ± 7	23	37	15
Acetylcholine	0.47 ± 0.1 (3)	8.3 ± 1.4	37	48	22
Methacholine	2.3 ± 0.4 (3)	35 ± 8.6	38	59	25

full agonist was similar to one of its two EC_{50} values for the cyclic nucleotide responses. The antagonist pirenzepine differentially inhibited the two responses to carbachol, with K_D values determined by analysis of the concentration response curves, differing by at least a factor of 10. All of the 10 agonists tested could reduce PGE_1-stimulated [^{3}H]cyclic AMP levels, but only five were able to increase significantly [^{3}H]cyclic GMP levels. The five partial agonists that differentiated between the responses by having little or no effect on [^{3}H]cyclic GMP levels were arecoline, bethanecol, McN-A343, oxotremorine, and pilocarpine. However, these partial agonists inhibited the [^{3}H]cyclic GMP responses to carbachol, indicating that they could bind to the low affinity receptor and suggesting that they could not induce the required conformational change to stimulate it. Their inhibition constants determined by the dose-ratio method agreed reasonably well with their equilibrium dissociation constants obtained by binding methods. The binding studies indicated that the partial agonists bound to all the muscarinic receptors with a single affinity constant. This binding constant approximated the EC_{50} for the reduction of [^{3}H]cyclic AMP levels.

If the partial agonists actually bind to two sites, they must do so with K_D values for the two sites that differ by less than 5- to 10-fold, or they must induce less than 10% of the second site, because these are the limits to the analytical technique. Interestingly, Birdsall and coworkers (22, 23) found that multiple sites for arecoline, pilocarpine, and oxotremorine were present in rat brain membranes, but for pilocarpine the ratio of affinities for the high and low affinity sites was reported as 1 or 4, and for oxotremorine it was 10 or 11. Thus, if N1E-115 receptors are like receptors in cerebral cortex, oxotremorine and pilocarpine would not easily differentiate them in the binding assay. In another study (24), it was noted that pilocarpine in N1E-115 cells did not stimulate cyclic GMP and its binding constant was actually evaluated by its inhibition of carbachol's stimulation. The published (22) value for the ratio of affinities for arecoline in brain homogenates is 87. However, in our hands, the binding assay with arecoline in rat brain homogenates gave $K_L/K_H = 17 \pm 3$ ($n = 8$).[2] Thus, it is possible that arecoline really does not largely differentiate between binding sites in N1E-115 cells. It is noteworthy that the dissociation constants for four partial agonists determined by the dose-ratio method independently from radioligand binding, gave K_D values reasonably similar to one-site binding K_D values (Table 4). Nevertheless, the important conclusion is that partial agonists were demonstrated to bind to the receptors mediating [^{3}H] cyclic GMP, without stimulating this response, and did so with K_D values quite similar to their EC_{50} values for stimulating the other response, implying that their inability to induce or detect a different, low affinity conformation accounts for their inability to elevate [^{3}H] cyclic GMP. At a concentration that would maximally reduce [^{3}H]cyclic AMP, the partial agonist occupied most of the sites involved with the [^{3}H]cyclic GMP elevation, but without this latter response occurring.

Heterogeneous populations of muscarinic receptors have been detected in various tissues but it has not been clear if these multiple receptors reside in the same cell or in different cells. In this study of muscarinic receptor heterogeneity, this interpretational difficulty was eliminated by employing a single cell type in culture, the well characterized murine neuroblastoma clone N1E-115. Data were obtained for two different responses in the same cell type, the intracellular molecular mechanisms of which are different. Therefore, our study directly addresses whether or not multiple muscarinic receptors are present to mediate the two different responses in the same cell.

The idea of muscarinic receptor heterogeneity is well accepted. Agonists detect multiple binding sites differentiated by affinity; the antagonist pirenzepine and certain others inhibit different responses *in vivo* with different potencies; and some agonists are able to stimulate certain responses better than others. Since it is likely that the receptor protein is basically the same in all tissues (25), the relevant question is how one receptor or binding protein can activate multiple effectors. If a binding protein could somehow become predisposed or committed to one effector or the other, it might split the receptor population into several classes. This commitment may occur by chemical modification or by coupling to another protein, as suggested by Birdsall and coworkers (22, 26), and such a modification could explain the different binding affinities for ligands and different EC_{50} values for agonists. The data for N1E-115 cells presented here support this concept of multiple sites or conformations present to mediate responses. Though binding heterogeneity could arise from either negative cooperativity between multimers of receptors or the presence of multiple independent sites with differing binding affinities, Birdsall's experiments (22) have indicated that at least in brain there is no cooperativity between receptors. In our previous study of N1E-115 receptors, we showed that the low affinity agonist site was selectively lost with multiple subculturing; the high affinity agonist site was maintained (10). This finding supports the con-

[2] M. McKinney, unpublished information.

cept of multiple independent sites better than cooperative binding sites. The steep or shallow Hill slopes (e.g., Fig. 5) for the responses cannot be taken alone as evidence for cooperative receptor interactions because of the possible presence of a nonlinear component in the intracellular mechanism which couples the receptor occupancy to the cyclic nucleotide level.

The response/inhibition data do not reveal whether the receptors are physically coupled to the effectors or if the receptor protein is identical in these two systems. However, the binding data with the full agonist and with pirenzepine do indicate that at equilibrium the receptors are distributed between two states or conformations. The similarity between binding equilibrium dissociation constants and EC_{50} values suggests that the binding heterogeneity reflects the response/inhibition heterogeneity. The full agonist can induce or detect two conformations; the partial agonist can induce one conformation. By "induce," we presume that the active agonist-receptor complex is a conformational state different from the ground state of the receptor protein(s). Pirenzepine blocks by inducing an inactive conformation of each class of receptors at much different (10-fold) concentrations. We suggest that the lower affinity agonist conformation mediates cyclic GMP formation, is effectively induced or revealed only by certain agonists (e.g., carbachol), and is inhibited by pirenzepine with high potency. Pirenzepine's potency at this site would classify it as an "M_1" response though the original classification of responses by this term was based on agonist efficacy (27). McN-A343, a drug that is M_1-specific in other systems (i.e., a ganglionic stimulator; Ref. 28), is not effective in N1E-115 cells in elevating cyclic GMP levels. (This raises the question of possible oversimplification of muscarinic receptor classification in various tissues by the use of one or a few "specific" drugs.)

There were six agonists that in the N1E-115 cells could stimulate both responses significantly: acetylcholine, bethanecol, carbachol, methacholine, muscarine, and *cis*-dioxolane. All these drugs contain the "NCCOCC backbone" (29) with the exception of bethanecol and carbachol which have an amine in place of the final methyl group. The four partial agonists that were unable to mediate the [^{3}H]cyclic GMP response were: arecoline, oxotremorine, pilocarpine, and McN-A343. These drugs do not contain this backbone but have larger and more complex structures. Our findings thus indicate that there are certain restrictive structural features of the agonist requisite for it to induce the conformation of the receptor appropriate for the [^{3}H]cyclic GMP response. The [^{3}H]cyclic AMP response, by contrast, was mediated effectively by all the agonists tested. The similarity between high affinity binding constants and the EC_{50} values suggests to us that the high affinity conformation mediates this response and is inhibited by pirenzepine with low potency (100–200 nM). This receptor subtype may not be the "M_2" receptor in the literature, for which pirenzepine seems to have a micromolar equilibrium dissociation constant (30). However, an intermediate binding site for pirenzepine (200 nM) has been identified as one of three sites in various tissues (31).

We have proposed that the N1E-115 muscarinic receptors might be precoupled to effectors; i.e., in the basal state they are already somehow predisposed or committed to either effector (2). This would give two receptor-effector complexes that could give rise to different binding affinities for the agonist (A) and for pirenzepine (see the diagram).

$$R_H \cdot E_A \underset{-A}{\overset{+A}{\rightleftharpoons}} A \cdot R_H \cdot E_A^*$$

$$\begin{array}{l|l} -E_A & -E_G \\ +E_G & +E_A \end{array}$$

$$R_H \cdot E_G \underset{-A}{\overset{+A}{\rightleftharpoons}} A \cdot R_L \cdot E_G^*$$

The binding protein would be essentially the same for either complex, but modified in some way in order for it to interact with a particular effector (E_A^* and E_G^* indicate activated effectors). The receptor-effector complexes are shown to be interconvertible, depending on the interchange of effectors. Full agonists would bind to the two receptor-effector complexes with different affinities. At higher concentrations of the full agonist, the low affinity ternary complex ($A \cdot R_L \cdot E_G^*$) would form in addition to the high affinity complex ($A \cdot R_H \cdot E_A^*$). For the full agonists, the low affinity complex lies about 2 kcal in energy above the high-affinity complex. Partial agonists, able to induce or detect a high affinity conformation only, would bind to both receptors with the same or nearly the same affinity. Something intrinsic to the receptor-effector structure and the partial agonist structure makes the lower affinity conformation unachievable. This model would require that the high affinity conformation is achievable even if the receptor is coupled to E_G. We are not yet certain whether the full agonist and pirenzepine detect a common feature of the receptor (such as the coupling mechanism) and thus give rise to identical relative proportions of binding sites. Additionally, the possibility exists that pirenzepine does not act in a simple competitive fashion.

If the receptors are mobile (not precoupled), then more complex schemes are required to account for the data. In particular, it is difficult to account for the one-site character of partial agonists and the selectivity of pirenzepine. In view of the fact that little of the receptor protein chemistry is known for muscarinic receptors, it is best to await such findings before speculating.

Fisher *et al.* (32) observed that acetylcholine, carbachol, methacholine, and muscarine bound to two muscarinic sites in brain synaptosomes and stimulated phospholipid turnover with EC_{50} values near the low affinity binding constant. Conversely, partial agonists bound to one class of sites, were much less able to mediate the response, and inhibited the response to carbachol. Their binding dissociation constants for the latter drugs (bethanecol, pilocarpine, arecoline, and oxotremorine) are remarkably similar to those reported in this paper. Brown and Brown (33) showed in chick heart slices that

carbachol mediated both a reduction of catecholamine-stimulated cyclic AMP increases and a turnover of phospholipids, but that oxotremorine was effective only in the former response and also inhibited carbachol's phospholipid turnover response. In a further study, Fisher and co-workers (34) showed that modifications of oxotremorine caused variations in efficacy in inositol lipid turnover in cerebral cortex and demonstrated that a correlation existed between efficacy and the degree to which a low affinity binding site was induced. Thus, there is some previous evidence that agonists differentiate between muscarinic responses, that partial agonists bind to a single class of muscarinic sites, and that full agonists induce a second site.

Our data provide some novel insights into muscarinic mechanisms of receptor-effector coupling. Most importantly, it is clear that, in neural cells at least, muscarinic agonists are not equally able to mediate responses at each receptor subtype. In fact, some may behave as antagonists at one response while behaving as agonists at another response. Given the variables of anatomic distribution of cholinergic systems in the central nervous system, subcellular localization of receptors (e.g., presynaptic versus postsynaptic), and function subserved, one can expect quite complex outcomes with administration of muscarinic drugs in pharmacotherapy of neuropsychiatric problems. With the central muscarinic receptor systems, a relevant issue is that of the administration of cholinomimetics in the treatment of Alzheimer's disease, in which central cholinergic systems are profoundly affected (35). Knowledge of the details of the molecular pharmacology of muscarinic receptor-effector coupling will allow increasing rigor and clinical confidence in selecting a therapeutic strategy for Alzheimer's disease. In relating the findings of this paper to this issue, one obvious conclusion that can be reached is that if a receptor similar to that mediating cyclic GMP in N1E-115 cells is the critically denervated cortical receptor, then it will be difficult to treat the disease with systemic administration of some agonists. All the agonists capable of effectively mediating cyclic GMP formation studied here (acetylcholine, carbachol, methacholine, *cis*-dioxolane, and muscarine) have charged nitrogens or have sensitive ester groups. It is not likely that substantial brain levels of these agonists could be achieved by systemic administration. A recent report showed, however, that intracranial cholinergic drug infusion in Alzheimer's disease patients is a promising means of activating brain muscarinic receptors (36). Near-term work could be directed at identifying selective agonists that can cross the blood-brain barrier and in particular at determining how the different muscarinic receptor subtypes function in the normal and the disease states.

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