0026-895X/88/060769-10$02.00/0

MOLECULAR PHARMACOLOGY, 34:769-778

Muscarinic Acetylcholine Receptors Linked to the Inhibition of Adenylate Cyclase Activity in Membranes from the Rat Striatum and Myocardium Can Be Distinguished on the Basis of Agonist Efficacy

MARY KEEN[1] and STEFAN R. NAHORSKI

Department of Pharmacology and Therapeutics, University of Leicester, Leicester LE1 9HN, United Kingdom

Received May 25, 1988; Accepted August 26, 1988

SUMMARY

Muscarinic agonists produce an inhibition of adenylate cyclase activity in broken cell preparations from rat striatum and myocardium. We have attempted to determine the occupancy-response relationships of three muscarinic agonists (carbachol, arecoline, and pilocarpine) by comparing their dose-response curves in these preparations with occupancy curves obtained under the same conditions. These comparisons suggest that the occupancy-response relationships for all three agonists in myocardium and for arecoline and pilocarpine in striatum are linear, response being directly proportional to occupancy. However, there appears to be a considerable receptor reserve for carbachol in the striatum, with carbachol producing a 50% maximal response at concentrations that occupy only 3% of the striatal receptors. These postulated occupancy-response relationships have been tested by blocking different proportions of the muscarinic receptors with the irreversible antagonist benzilylcholine mustard. The effects of this blockade on the dose-response curves are as predicted from the occupancy-response relationships, suggesting that these relationships are correct. The relative efficacies of these muscarinic agonists can be determined from their occupancy-response relationships. The efficacies of arecoline and pilocarpine relative to carbachol are ~0.13 and ~0.03 in striatum and ~1.0 and ~0.45 in myocardium, respectively. This difference in relative efficacies in the two tissues is evidence of a real conformational difference between the muscarinic receptors linked to adenylate cyclase in these preparations.

The elucidation of the precise relationship between the binding of an agonist to its receptor and the initiation of a functional response is one of the most fundamental problems in pharmacology. Muscarinic agonists are capable of producing a functional response, inhibition of adenylate cyclase activity, in broken cell preparations from a number of tissues (1–3). We have used the muscarinic inhibition of adenylate cyclase activity in membrane preparations from the rat striatum and myocardium as simple model systems in which it should be possible to directly compare agonist binding and response.

There is increasing evidence for the existence of subtypes of the muscarinic acetylcholine receptor. The selective antagonists pirenzepine (4) and AF-DX-116 (5) exhibit different affinities for the muscarinic receptors in forebrain and myocardium, and genetic studies have shown that these receptors seem to have different primary amino acid structures (6). It has been widely assumed, however, that most muscarinic agonists exhibit little or no selectivity between any of the postulated subclasses of muscarinic receptor and even the selectivity of the prototypical selective muscarinic agonist McNA343 (7) has been questioned (8).

The apparent lack of selectivity of muscarinic agonists is perhaps rather surprising. The action of an agonist involves two processes, the initial binding of the agonist to the ground state of the receptor and the subsequent activation of the agonist-receptor complex to initiate a response, and is governed by two receptor specific parameters, affinity and efficacy (9, 10). It might thus be expected that agonists will be rather more sensitive to subtle differences in receptor-structure or receptor-effector molecule interactions than are antagonists, the action of which depends solely on their affinity for the receptor. However, it is difficult to determine agonist affinity and efficacy by classical pharmacological methods (11) and their potential for receptor classification has not been fully exploited (10).

[1] Present address: Department of Pharmacology, University of Birmingham, Medical School, Vincent Drive, Birmingham B15 2TJ, United Kingdom.

ABBREVIATIONS: BCM, benzilylcholine mustard; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetra acetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulphonic acid; IBMX, 3-isobutyl-1-methyl xanthine; NMS, N-methylscopolamine; *p*, fractional occupancy; SDS, sodium dodecyl sulfate.

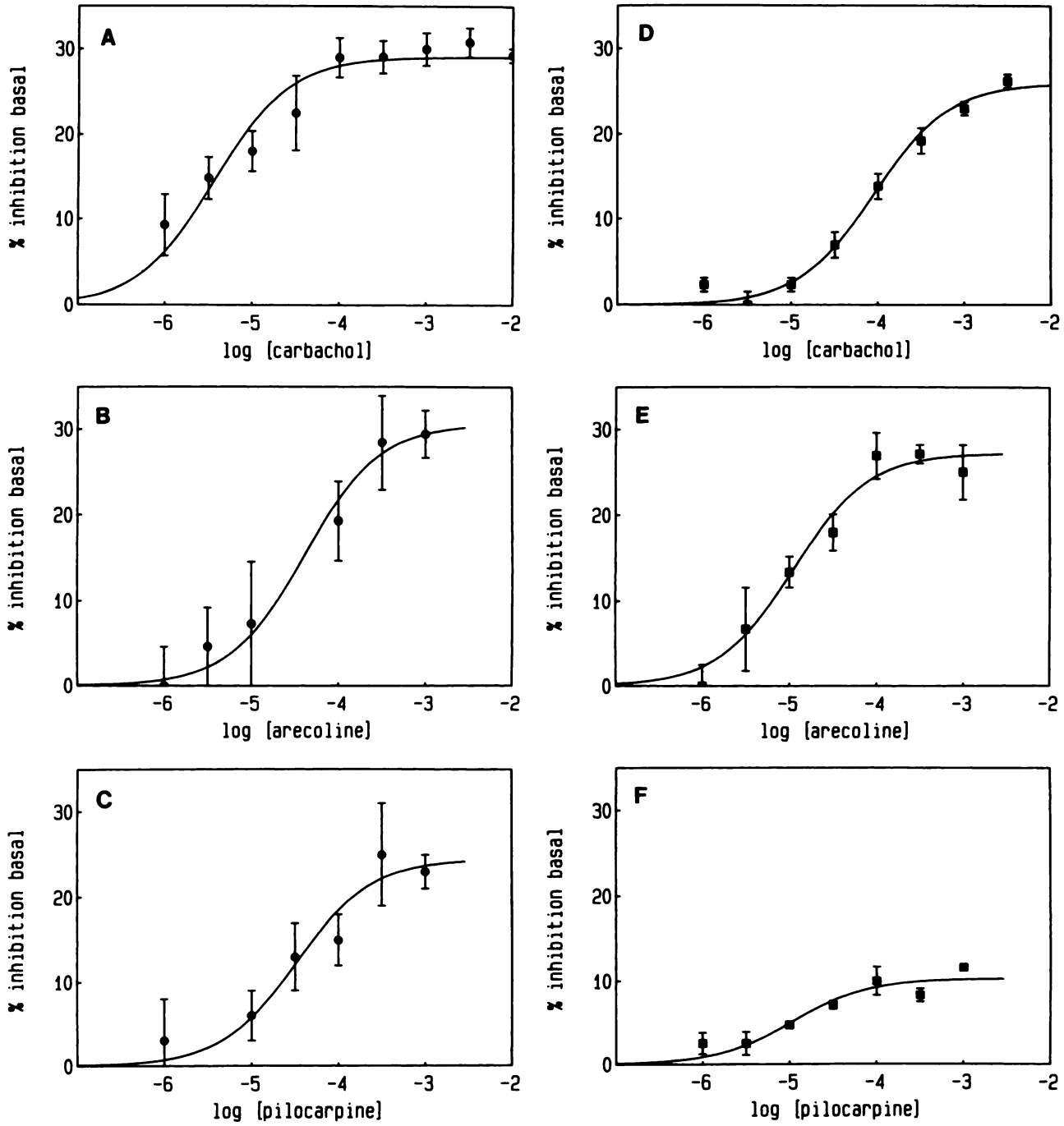

Fig. 1. Inhibition of adenylate cyclase activity in rat striatal (A–C) and myocardial (D–F) membranes by carbachol, arecoline, and pilocarpine. These are single, representative dose-response curves, each *point* representing the mean ± standard error per cent inhibition of basal adenylate cyclase activity determined as described in Materials and Methods. The *solid lines* represent the best fit of simple Langmuir isotherms ($n_H = 1$) to the data. Basal activity was as stated in Table 1.

In this study we have attempted to determine muscarinic agonist affinity and efficacy from comparison of agonist binding curves with dose-response curves obtained under exactly the same conditions.

Materials and Methods

Membrane Preparation

Striata and hearts were obtained from male Wistar rats (200–300 g), killed by decapitation. Homogenates from these tissues were prepared as follows.

Striatum. Striata were dissected out on ice and homogenized in 20 volumes of ice-cold homogenization buffer (20 mM HEPES/2 mM EGTA, pH 7.4) in a motor-driven Teflon-glass tissue grinder. The homogenate was centrifuged at 48,000 × *g* for 15 min and the pellet was resuspended in the supernatant volume of homogenization buffer. This suspension was then stirred on ice for 30 min to ensure lysis of any sealed membrane vesicles. This preparation was then either used immediately in the cyclase or binding assays, treated with irreversible antagonist, or frozen at −40°. Preliminary experiments showed that membranes could be frozen for up to three weeks without any apparent effects on binding or adenylate cyclase activity.

TABLE 1

Maximum responses and EC_{50} values for the inhibition of adenylate cyclase and binding affinities (K) of three muscarinic agonists in striatal and myocardial membranes

Binding affinity values were obtained from analysis of agonist binding curves performed in the presence of guanine nucleotide, i.e., conditions that correspond to those of the cyclase assay. Maximum responses and EC_{50} values were obtained from analysis of dose-response curves for the inhibition of adenylate cyclase activity in the two membrane preparations. Basal cyclase activities were typically 10–20 pmol of cAMP/mg of protein/min in striatal membranes and 1–3 pmol of cAMP/mg of protein/min in myocardial membranes. Values are mean ± standard error for three to five determinations.

	Maximal response	log EC_{50}	log K
	% inhibition of basal activity		
Striatum			
Carbachol	23.0 ± 3.9	−5.19 ± 0.12	−3.62 ± 0.08
Arecoline	24.1 ± 2.8	−4.82 ± 0.20	−4.21 ± 0.27
Pilocarpine	17.9 ± 1.9	−4.55 ± 0.91	−5.17 ± 0.24
Myocardium			
Carbachol	24.6 ± 1.4	−4.39 ± 0.19	−4.45 ± 0.06
Arecoline	24.7 ± 0.6	−4.89 ± 0.07	−4.61 ± 0.08
Pilocarpine	10.8 ± 1.2	−5.10 ± 0.10	−5.00 ± 0.25

Heart. Hearts were perfused *in situ* with ice-cold homogenization buffer and excised, and ventricles and atria were minced with scissors and homogenized in 10 volumes of homogenization buffer using a Polytron. The homogenate was filtered through a single layer of cheesecloth and then centrifuged at 48,000 × *g* for 15 min. The pellet was resuspended in the supernatant volume of homogenization buffer, using a Teflon-glass tissue grinder. The suspension was then stirred on ice and used exactly as described for the striatal preparation.

Irreversible Antagonist Treatment

Membrane preparations were incubated at 37° for up to 10 min in the absence (for controls) or presence of the alkylating muscarinic antagonist BCM (12). The BCM solution had previously been incubated in 20 mM HEPES, pH 7.4, for 60 min at room temperature, to allow formation of the active aziridinium ion. The concentrations of BCM used were nominally (i.e., total but not necessarily active) 10 nM for the striatal membranes and 100 nM for the heart membranes, which proved rather more resistant to alkylation. Different degrees of receptor blockade were achieved by incubating the membranes with the BCM for different lengths of time. After the incubation period, the mixture was diluted with ice-cold homogenization buffer and immediately centrifuged at 48,000 × *g* for 15 min. Free BCM was removed by three subsequent centrifugation and resuspension steps. The BCM-treated membranes were routinely frozen in aliquots at −40° before use in the binding or adenylate cyclase assays.

Adenylate Cyclase Assays

Adenylate cyclase assays were performed by one of two methods, both of which are modifications of the method described by Olianas *et al.* (2). Despite minor differences, both assays gave apparently identical results.

Binding protein method. Membranes (0.2–0.3 mg of protein) were incubated in a final volume of 250 μl, containing 4 mM HEPES, 64 mM Tris maleate (pH 7.4), 0.5 mM EGTA, 100 mM NaCl, 3.2 mM $MgCl_2$, 1 mM IBMX, 0.1 mM GTP, 1 mM ATP, and muscarinic agonists as described in Results. The assay (performed in quintuplicate) was started by the addition of the ATP, after preequilibrating the membranes with the agonists and other assay components for 15 min at 0°. The samples were incubated for 3 min at 37°, and the reaction was then terminated by plunging the tubes into a boiling water bath for an additional 3 min. Preliminary experiments had shown that the rate of cAMP formation was linear for up to 5 min. The samples were centrifuged (1500 × *g* for 15 min) and the amount of cAMP in the supernatant was determined by a cAMP protein binding assay (13).

[α-^{32}P]ATP Method. Membranes (~0.4 mg of protein of the heart preparation or ~0.1 mg of protein of the striatal preparation) were incubated in a final volume of 50 μl, containing 4 mM HEPES, 50 mM Tris-HCl (pH 7.4), 0.5 mM EGTA, 100 mM NaCl, 4 mM $MgCl_2$, 400 μM cAMP, approximately 20,000 cpm of [^{3}H]cAMP, 0.33 mM dithiothreitol, 5 mM creatine phosphate, 50 units/ml creatine phosphokinase, 1 mM IBMX, 0.1 mM GTP, 0.5 mM ATP, approximately 3,000,000 cpm of [α-^{32}P]ATP, and muscarinic agonists as stated in Results. As above, the assay (performed in triplicate) was started by addition of the ATP (including the [α-^{32}P]ATP) after preincubation of the membranes on ice with the rest of the assay components. The samples were incubated for 15 min at 37°, inasmuch as preliminary experiments had shown that the time course of cAMP formation was linear for over 20 min under these conditions. The reaction was stopped by the addition of 100 μl of stopping solution (2% SDS, 45 mM ATP, 1.3 mM cAMP). The amount of [^{32}P]cAMP formed was measured by liquid scintillation counting after separation of cAMP and ATP by the method of Salomon *et al.* (14) on Dowex and alumina columns. Recovery of [^{3}H]cAMP was typically 85–95%.

Binding Assays

Muscarinic receptors were defined as the atropine (1 μM)-displaceable binding of [^{3}H]NMS (0.03–20 nM). Membranes were incubated with [^{3}H]NMS and other muscarinic drugs as stated in Results, in a medium containing either 4 mM HEPES, 64 mM Tris maleate (pH 7.4), 0.5 mM EGTA, 100 mM NaCl, 3.2 mM $MgCl_2$, 0.1 mM GTP, and 1 mM ATP (for comparison with the binding protein cyclase assay) or 4 mM HEPES, 50 mM Tris-HCl (pH 7.4), 0.5 mM EGTA, 100 mM NaCl, 4 mM $MgCl_2$, 400 μM cAMP, 0.33 mM dithiothreitol, 0.1 mM GTP, and 0.5 mM ATP (for comparison with the [^{32}P]ATP cyclase assay). These media were the same as those used in the adenylate cyclase assays, with the exception of the ATP-regenerating system and the IBMX, neither of which had any effect on binding. The membrane preparations were diluted with homogenization buffer, where appropriate, before addition to the assay, to avoid undue depletion of the [^{3}H]NMS by receptor-specific binding. For the studies on the effects of guanine nucleotides on agonist binding, all the nucleotides were omitted from the incubation medium, in case they contained any GTP as a contaminant; 10^{-4} M GppNHp was routinely used in place of GTP in these studies to avoid any problems due to hydrolysis of GTP. Incubations were routinely performed for 30 min at 22° because this yielded exactly the same results as the incubation conditions used in the adenylate cyclase assays, i.e., 15 min at 0°, then 3 min, or 15 min at 37°. Bound and free [^{3}H]NMS were separated either by centrifugation (in which case the final incubation volume was 1 ml) or by filtration, using a Brandel cell harvester (final volume was then 250 μl); again, these two methods gave apparently identical results. All assays were performed in duplicate.

Analysis of Data

Dose-response and binding curves were analyzed by ALLFIT (15). When the Hill coefficients of the agonist binding curves were less than 0.8, these curves were analyzed by computer-assisted nonlinear regression analysis to a two independent binding site model (16). All agonist affinities were corrected for any shift in the binding curve caused by the occupancy of [^{3}H]NMS (17).

Results

Muscarinic inhibition of adenylate cyclase. Typical dose-response curves obtained for the inhibition of basal adenylate cyclase activity by three muscarinic agonists (carbachol, arecoline, and pilocarpine) in membranes from rat striatum and myocardium are shown in Fig. 1. The pooled results from a number of these experiments are listed in Table 1. All the dose-response curves could be well described by simple Langmuir isotherms (i.e., they have Hill coefficients of 1). In both preparations, carbachol and arecoline produced the same maximal responses (see Table 1) and thus appear to be full agonists.

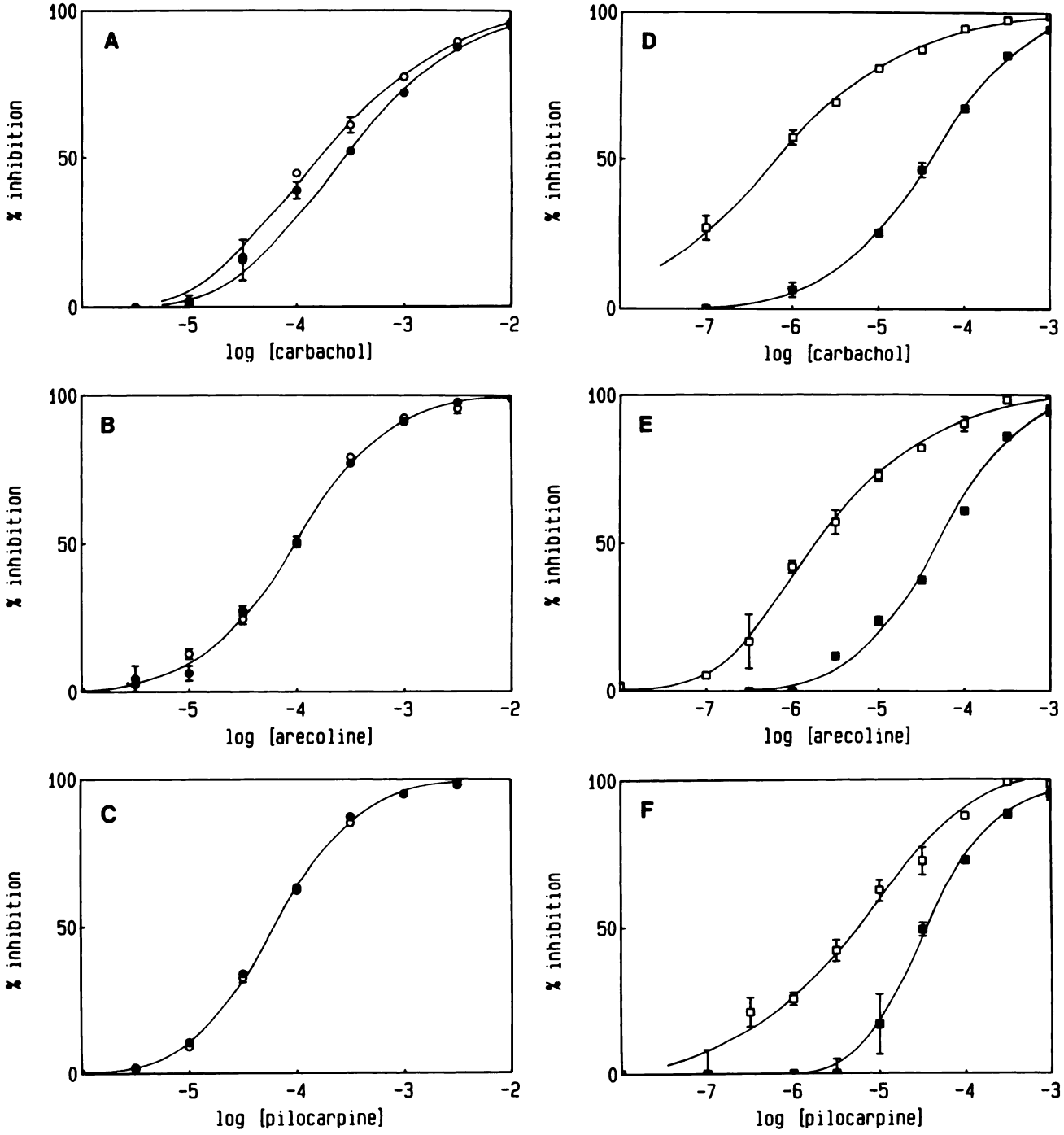

Fig. 2. Inhibition of specific [^{3}H]NMS binding to rat striatal (A–C) and myocardial (D–F) membranes by carbachol, arecoline, and pilocarpine in the absence (*open symbols*) and presence (*closed symbols*) of 10^{-4} M GppNHp. These are single, representative binding curves, each *point* representing the mean ± standard error per cent inhibition of the specific binding of a tracer concentration of [^{3}H]NMS determined as described in Materials and Methods. These curves are not corrected for [^{3}H]NMS occupancy.

Pilocarpine behaved as a partial agonist for the inhibition of adenylate cyclase activity in both membrane preparations, with a relative intrinsic activity (i.e., maximal response to a partial agonist relative to the maximal response produced by full agonists) of 0.75 in striatum and 0.44 in myocardium. Comparison of the EC_{50} values for the various agonists in the two preparations show that the carbachol dose-response curve in striatal membranes lies to the left of the carbachol dose-response curve in the myocardial preparation.

Agonist binding properties. The aim of this study was to compare agonist dose-response curves with agonist binding curves obtained under the conditions used in the functional assay. Thus, binding experiments were routinely performed in the presence of guanine nucleotide (see Materials and Methods). However, the effects of guanine nucleotides on the agonist binding properties of the striatal and myocardial membrane preparations were briefly examined, and Fig. 2 shows agonist binding curves in the absence and presence of guanine nucleo-

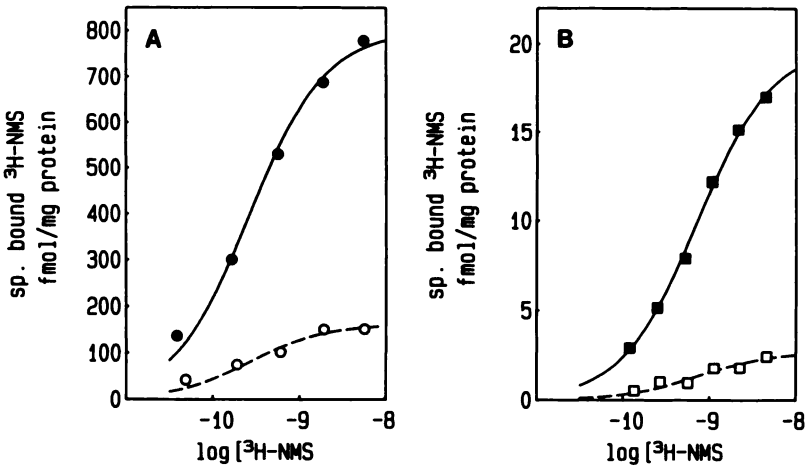

Fig. 3. [³H]NMS saturation binding curves to control (*closed symbols*) and BCM-treated (*open symbols*) membranes from rat striatum (A) and myocardium (B). The *curves* represent the best fit of simple Langmuir isotherms (n_H = 1) to the data, with the affinity of [³H]NMS (K) constrained to be the same in the control and treated preparations. K = 0.27 nM in striatum and K = 0.73 nM in myocardium.

TABLE 2

Affinities (K) and maximal binding capacity (B_{max}) of [³H]NMS in control and BCM-treated membranes from the rat striatum and myocardium

Affinities and B_{max} values were obtained from analysis of [³H]NMS saturation binding curves (see Materials and Methods). Values are mean ± standard error for three to five determinations.

	Striatum	Myocardium
B_{max} (control) (pmol/mg of protein)	0.72 ± 0.05	0.021 ± 0.001
log K (control)	−9.54 ± 0.04	−9.26 ± 0.12
log K (BCM-treated)	−9.64 ± 0.04	−9.24 ± 0.10

tide (the effects of 10^{-4} M GTP and 10^{-4} M GppNHp on agonist binding were indistinguishable under these conditions).

In general, in the presence of guanine nucleotides, the Hill slopes of the agonist binding curves approach 1 (18). This suggests that under these conditions the receptors are fairly homogeneous with regard to agonist affinity and that any estimate of agonist affinity obtained from these curves (which assay the total population of muscarinic receptors, labeled with [³H]NMS) will provide at least an approximation of the agonist affinity for that subpopulation of the receptors in the two membrane preparations that are coupled to the inhibition of adenylate cyclase.

Occasionally, however, there was some evidence for heterogeneity of agonist binding sites even in the presence of guanine nucleotide. This was particularly noticeable with carbachol (see Fig. 4a), which, as the most efficacious agonist used, is expected to exhibit the most selectivity between high and low agonist affinity states of the receptor (18). This apparent deviation from homogeneity seems to be due to the existence of a small proportion (less than 10%) of sites that retain high agonist affinity even in the presence of 10^{-4} M GTP or GppNHp (e.g., the carbachol binding curve in control striatal membranes shown in Fig. 4A can be well described by a model in which 10% of sites have $K = 1.0 \times 10^{-6}$ M and 90% have $K = 1.2 \times 10^{-4}$ M). The significance of these apparently GTP-insensitive sites remains obscure. It has been suggested that they may occur as a result of proteolysis of the receptor and/or the N-protein, which renders the complex insensitive to GTP (19) and possibly, therefore, inactive in mediating any response. There was no correlation between the appearance (or nonappearance) of these sites and any apparent effects on the dose-response relationships for any of the agonists in the functional assay on the same membrane preparations. Where heterogeneity was apparent ($n_H < 0.8$), fitting the agonist binding curves to a two-site model yielded affinity values for the low affinity site that were indistinguishable from the agonist affinities obtained from analysis (by a one-site model) of the apparently homogeneous agonist binding curves ($n_H > 0.9$). These agonist affinities, obtained under the same conditions as the functional assay, are shown in Table 1.

Effects of BCM. Incubation of the striatal and myocardial membrane preparations with the irreversible antagonist BCM, as described in Materials and Methods, resulted in a time-dependent decrease in the number of available muscarinic receptors, as defined by the atropine (1 μM)-displaceable binding of [³H]NMS (0.03–20 nM).

The BCM treatment had no apparent effect on the antagonist binding properties of the remaining unalkylated receptors, resulting in a decrease in the B_{max} of [³H]NMS saturation curves, with no effect on [³H]NMS affinity (Fig. 3; Table 2).

The degree of blockade obtained tended to be rather variable (e.g., on different days a 5-min treatment could produce 10–80% receptor loss) and it was not possible to predict the effect of any particular BCM treatment on receptor number. For these reasons, the 'loss' of receptors in any particular membrane preparation was always determined independently as the loss in [³H]NMS binding sites. Control levels of [³H]NMS binding sites in these membranes are shown in Table 2. The reason for the variation in the effects of BCM are not clear, but it is probably due to variation in the concentration of active (i.e., cyclized) BCM.

The agonist binding properties of the unalkylated receptors remain largely unaltered by the BCM treatment (Fig. 4). There was, however, some tendency for an increase in the proportion of apparently GTP-insensitive high affinity agonist binding sites that could be detected with carbachol in the striatal membranes (Fig. 4A). This was probably due to the relative resistance of these sites to alkylation by the BCM, as has been reported for high affinity agonist binding sites with respect to alkylation by the closely related irreversible antagonist propylbenzilylcholine mustard (20).

Dose-response curves for the various agonists were obtained in membranes in which a known proportion of the muscarinic receptors had been blocked by BCM. For arecoline and pilocarpine in striatal membranes, and all three agonists in myocardial membranes, BCM treatment results in a decrease in the max-

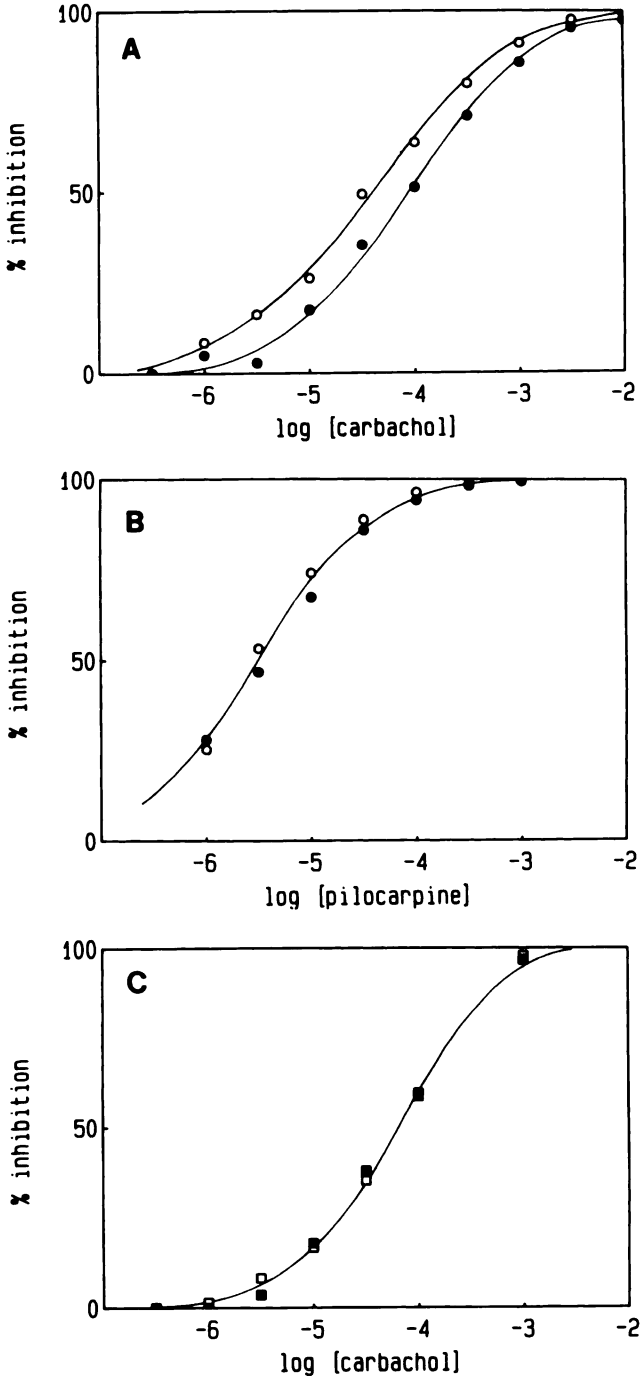

Fig. 4. Agonist binding curves in control (*closed symbols*) and BCM-treated (*open symbols*) membranes from rat striatum (A, B) and myocardium (C). Inhibition of the specific binding of [^{3}H]NMS by carbachol (A) and pilocarpine (B) in striatal membranes and by carbachol in myocardial membranes (C). The loss of receptors produced by the BCM treatment was 50%, 35%, and 25% in Fig. 5, A, B, and C, respectively. These curves are not corrected for [^{3}H]NMS occupancy.

imal response, directly proportional to the loss of receptors (Fig. 5). As the maximal response is decreased, it becomes harder to determine accurate EC_{50} values; however, the results were entirely consistent with there being no change in EC_{50} value for any of these agonists after BCM blockade.

Fig. 6A shows the dose-response curve for carbachol obtained in BCM-treated striatal membranes compared with its dose-response curve in control membranes; blockade of 50% of the receptors with BCM resulted in a small shift to the right of the dose-response curve, with clearly no decrease in the maximal response. This suggests the existence of a receptor reserve for carbachol in striatal membranes and is consistent with the fact that its dose-response curve lies well to the left of its binding curve (Table 1). Fig. 6B shows a dose-response curve for carbachol in BCM-treated myocardial membranes, in which there was no apparent receptor reserve, and in which BCM blockade led to a decrease in maximal response, with no apparent change in EC_{50} (see above).

Occupancy-response relationships. The results of the BCM experiments suggest that it *is* possible to estimate valid occupancy-response relationships from a direct comparison of agonist dose-response and binding curves. These occupancy-response curves are shown in Fig. 7. The occupancy-response relationships for carbachol and arecoline in myocardial membranes are shown as being identical (Fig. 7B), inasmuch as they cannot be distinguished using the available data. However, it seems likely that they are in fact slightly different, with carbachol having somewhat higher efficacy than arecoline.

The relative efficacies of the various agonists can be determined from these occupancy-response relationships, as described by Furchgott (21), as the antilog of the displacement of the curves along the log p axis, where p is fractional occupancy. The relative efficacies of the three agonists used here, in the two membrane preparations, are shown in Table 3. There are marked differences in the relative efficacies of the various agonists between the striatal and myocardial membrane preparations.

Discussion

According to the theory of agonist action formulated by Stephenson (9) and modified by Furchgott (21), the response produced by an agonist (A) is a function both of its affinity (K) for the ground [i.e., unactivated (22)] state of the receptor and its intrinsic efficacy (E) such that

$$\text{response} = f\left(\frac{[A]}{K + [A]}\, E\right)$$

The results of this study strongly suggest that valid estimates of affinity and efficacy can be obtained by comparing agonist binding and response. Muscarinic receptors are part of the large group of receptors, the action of which is mediated by the activation of guanine nucleotide-binding G proteins (23). For these receptors, it seems probable that K will represent the affinity of the agonist for the uncoupled receptor (R), and E will be the equilibrium governing the interaction of the agonist-receptor complex with the G protein (G), by which G is activated and a response generated. It may therefore be possible to determine agonist affinity and efficacy by analyzing agonist binding data using the ternary complex model:

$$\begin{array}{ccc} A + R + G & \overset{K}{\longleftrightarrow} & AR + G \\ \alpha E \downarrow & & \downarrow E \\ A + RG & \longleftrightarrow & ARG \\ & \alpha K & \end{array}$$

where affinity is K and efficacy is E (24).

Ehlert (24) has recently proposed that muscarinic agonist

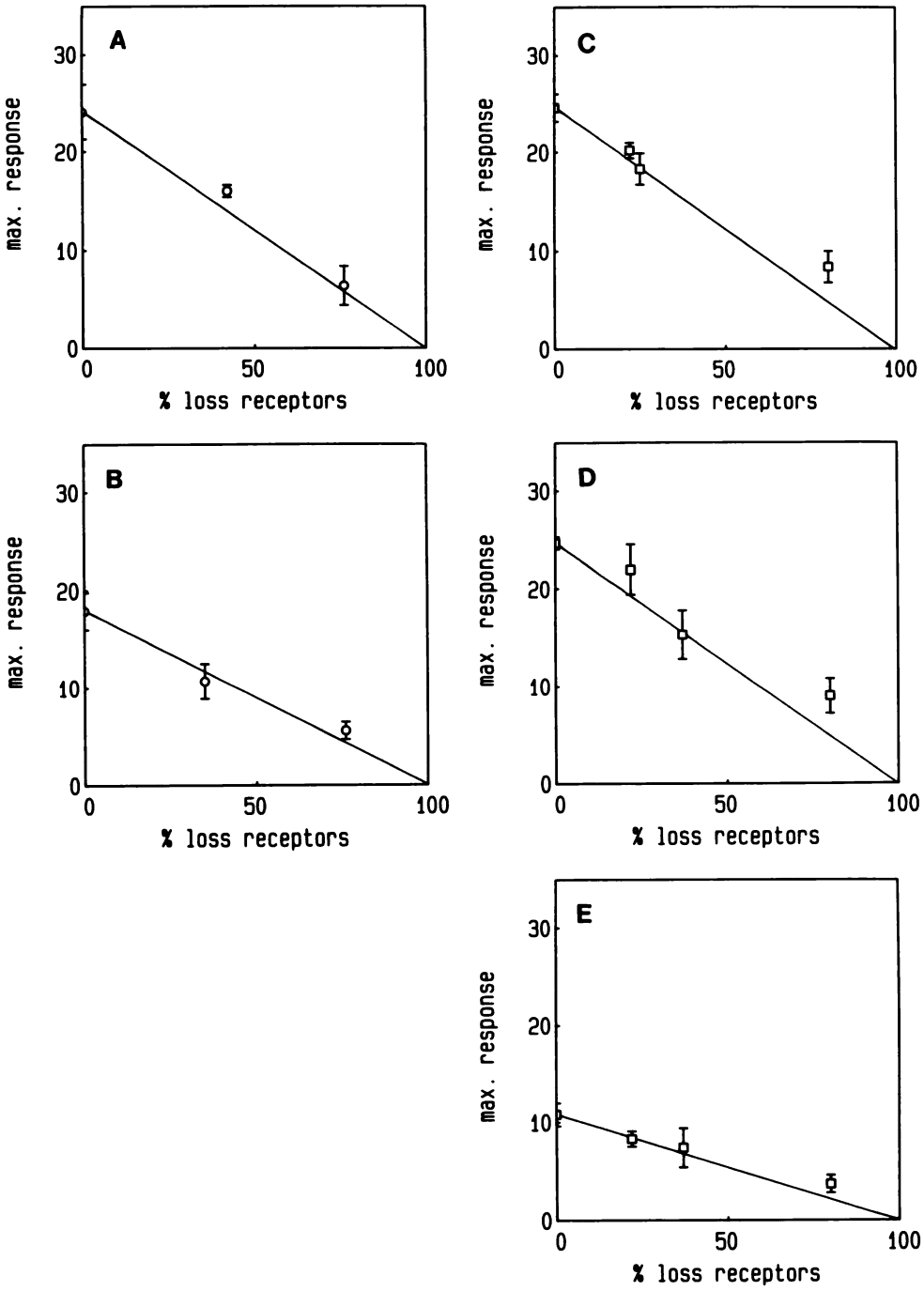

Fig. 5. Correlation of the per cent loss of muscarinic receptors after BCM treatment of striatal (A, B) and myocardial (C, D, and E) membranes with the maximal response (as per cent inhibition of basal adenylate cyclase activity) obtained with carbachol (C), arecoline (A and D) and pilocarpine (B and E) in these membranes. The *solid lines* represent the predicted relationship inferred from the maximal responses obtained for each agonist in control membranes (Table 1), assuming a linear relationship between binding and response.

binding to rabbit myocardial membranes can be well described by the ternary complex model and that the efficacy factor thus determined correlates well with the efficacy determined for inhibition of adenylate cyclase in the same preparation. However, his analysis depends on very little *G* being precoupled to *R* in the absence of agonist and this is inconsistent with evidence from solubilization studies (25), which show that, at least in rat myocardium, substantial amounts of *RG* complexes exist in the membrane in the absence of agonist.

In this study, no detailed analysis of the agonist binding curves has been attempted. However, the effects of guanine nucleotides and the apparent correlation between the magnitude of the GTP shift and agonist efficacy [carbachol, the most efficacious agonist used in this study, exhibits the largest GTP shift in both preparations; arecoline exhibits a smaller shift; and pilocarpine, a partial agonist in both preparations, exhibits the smallest shift of all (Fig. 2)] are broadly consistent with the predictions of the ternary complex model (18). There is a striking difference in the magnitude of the effect of guanine nucleotides on agonist binding curves between the striatal and myocardial preparations, with much greater effects apparent in the myocardium. Initially this is perhaps surprising, as the receptors in the striatal preparation seem to be rather more efficiently coupled to the inhibition of adenylate cyclase activity than are those in the myocardial preparation, as evidenced by the lower EC_{50} for carbachol and the higher relative intrinsic activity of pilocarpine in striatum. However, the GTP shift is related to the intrinsic efficacy of each individual agonist-

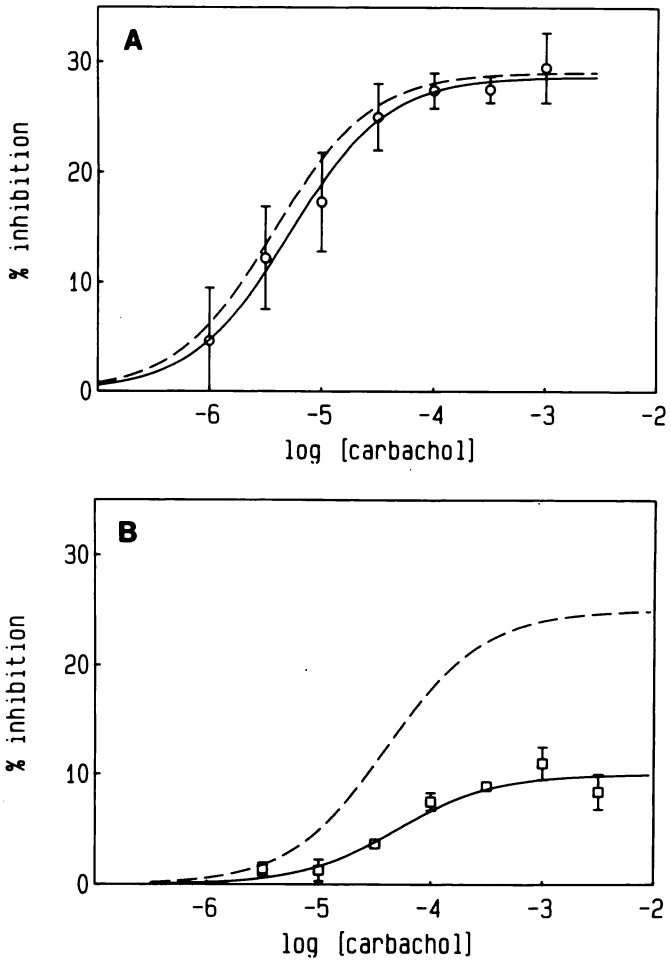

Fig. 6. Carbachol dose-response curves for the inhibition of adenylate cyclase activity in BCM-treated striatal (A) and myocardial (B) membranes. The *solid lines* represent the best fit of simple Langmuir isotherms (n_H = 1) to the data. The *dashed lines* represent the carbachol dose-response curves obtained in control membranes (see Table 1). The loss of receptors caused by the BCM treatment was 50% and 70% in striatum and myocardium, respectively. Basal adenylate cyclase activity was as stated in Table 1.

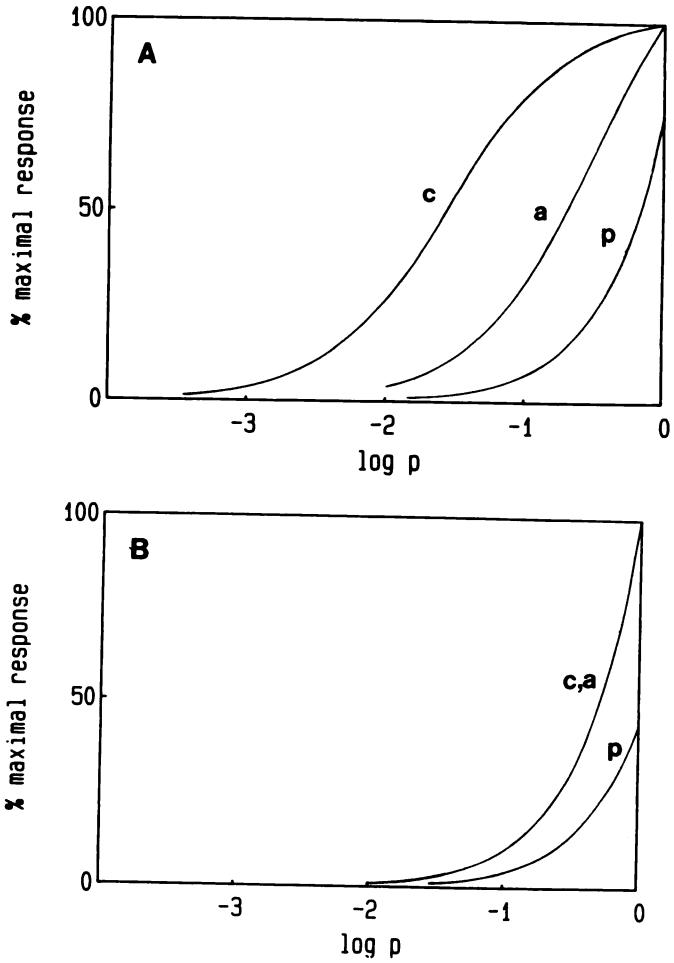

Fig. 7. Occupany-response curves for the inhibition of adenylate cyclase activity by carbachol (*c*), arecoline (*a*), and pilocarpine (*p*) in membranes from the rat striatum (A) and myocardium (B). Response (as a percentage of the maximal response to carbachol, a full agonist) is plotted against the log of the fractional occupancy, *p*, for each agonist, calculated from the data in Table 1.

receptor interaction, whereas the efficiency of the coupling of receptor occupany to response is a tissue-dependent phenomenon, depending, for example, on the total number of receptors (22). There is no *a priori* reason to expect any relationship between the two.

The validity of the estimates of agonist affinity and relative efficacy obtained in this study depends absolutely on the agonist binding properties observed in the binding assay (which measures *all* the muscarinic receptors in the membrane preparations) being relevant to that subpopulation of these receptors that are linked to the inhibition of adenylate cyclase activity, presumably by activation of G_i. It is quite clear that not all the muscarinic receptors in either striatum or heart are linked to adenylate cyclase inhibition; some are linked to phosphoinositide hydrolysis (26, 27) and some to the gating of ion channels (28, 29), and both these latter responses seem to involve activation of G proteins that are distinct from G_i (28, 30). It is also apparent that muscarinic receptors in striatum are heterogeneous with regard to their affinity for the selective antagonist pirenzepine (31). Fortunately for this study, these different populations of receptors within the striatum or myocardium all

TABLE 3

Relative efficacies of muscarinic agonists for the inhibition of adenylate cyclase activity in striatal and myocardial membranes

Relative efficacies were determined as the antilog of the displacement of the occupancy-response curves, shown in Fig. 8, along the log *p* axis, i.e., relative efficacy is the relative occupancy of two agonists when they are producing the same response.

	Relative efficacy
Striatum	
Arecoline/carbachol	0.13
Pilocarpine/carbachol	0.03
Pilocarpine/arecoline	0.23
Myocardium	
Arecoline/carbachol	1
Pilocarpine/carbachol	0.45
Pilocarpine/arecoline	0.45

seem to exhibit the same, or very similar, affinities for agonists in the presence of guanine nucleotide, as the Hill slopes of the agonist binding curves obtained under these conditions are generally very close to one. Perhaps this is because agonist binding measured under these conditions will be predominantly to receptors *not* coupled to G protein; receptors coupled to different G proteins might well be expected to exhibit different agonist affinities, but this would only be apparent in the ab-

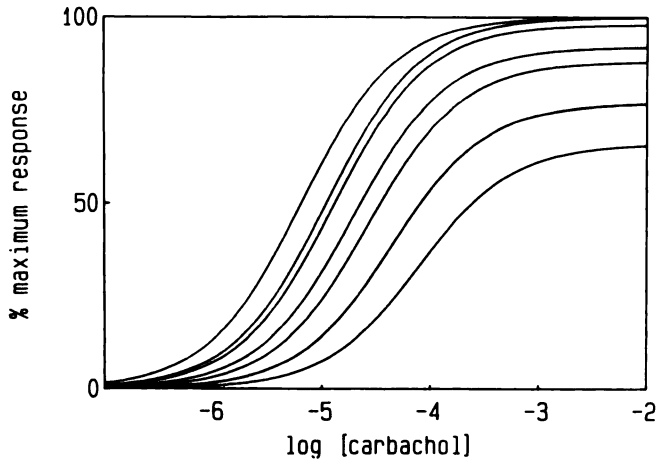

Fig. 8. Theoretical dose-response curves for the inhibition of adenylate cyclase by carbachol in rat striatal membranes after blockade of different proportions (0, 40, 50, 70, 80, 90, and 95%) of the available receptors. These curves were calculated from the control dose-response curve and the apparent carbachol affinity (Table 1) using the assumption of Furchgott and Bursztyn (11) (see text).

sence of guanine nucleotide. This type of phenomenon may explain why it can be difficult to fit muscarinic agonist binding data to simple two-site binding models (17).

Comparison of the dose-response and binding curves suggests that a simple linear relationship between occupancy and response exists for all three agonists studied in the myocardial preparation and for arecoline and pilocarpine in striatal membranes. If these apparent relationships between agonist binding and response are valid, then it should be possible to predict the effects of reducing the number of available muscarinic receptors, with an irreversible antagonist, on the agonist dose-response curves. For example, a 50% reduction in the number of available receptors would be expected to produce a 50% decrease in the maximal response to those agonists for which the response seems to be linearly related to agonist binding, with no change in their EC_{50} values. These predictions are borne out experimentally, suggesting that the inferred occupancy-response relationships are correct.

A considerable receptor reserve seems to exist for carbachol in the striatal preparation, with carbachol producing a 50% maximal response at concentrations that occupy only about 3% of the available receptors. Blockade of 50% of the available receptors with BCM leads to a small shift to the right of the carbachol dose-response curve, with no decrease in maximal response. It is possible to predict the effects of receptor blockade on the carbachol dose-response curve, using the assumption of Furchgott and Bursztyn (11) that, for any given agonist, equal responses before and after receptor inactivation must result from occupation of equal *numbers* of receptors. The predicted dose-response curves are shown in Fig. 8 and seem to agree with the data obtained for carbachol (Fig. 6A).

The curves shown in Fig. 8 raise some interesting questions about the relationship between binding and response. They show the predicted effect of reducing receptor *number*, while agonist affinity and efficacy remain constant. However, under the operational model of agonist action (32), exactly the same family of curves should be generated by keeping agonist affinity and receptor number constant while altering agonist efficacy, i.e., exactly the same function should be applicable to changes in receptor number as to changes in intrinsic efficacy. As has been pointed out by Colquhoun (22), the validity of the widely used irreversible antagonist method to determine the affinity of a full agonist in a functional assay (11, 21, 33) depends absolutely on these functions being identical. In the striatal membranes, however, this seems not to be the case. Reducing receptor number is predicted to result in a suppressed maximal response to carbachol when the EC_{50} is still well below the binding affinity (Fig. 8). However, the dose-response curve for arecoline (which has a lower intrinsic efficacy than carbachol) is apparently coincident with its binding curve while still producing a maximal (i.e., 'full agonist') response. It would be inappropriate to attempt to formulate a relationship between occupancy, intrinsic efficacy, and response from the data available here, but this tends to bear out Colquhoun's assertion that it is entirely arbitrary to assume that the effects of reducing receptor number or agonist intrinsic efficacy will be the same (22).

The existence of a receptor reserve usually implies an amplification step between agonist binding and the observed response. In the broken cell preparations studied here, the steps between receptor occupation and the observed inhibition of cyclase activity are presumably the activation of G_i and the subsequent interaction of that G_i with G_s and/or the adenylate cyclase molecule itself. It seems likely that amplification takes place at the level of G_i activation, and it is possible that the apparent 'receptor reserve' for carbachol in the striatal membranes is in fact an 'G_i reserve,' related to the large amounts of G_i that may be present in these membranes (23). It would be interesting to examine the effects on the carbachol dose-response curve of reducing the amount of functional G_i by treatment with pertussis toxin (34). However, considerable practical difficulties have been encountered in obtaining quantitative effects of pertussis toxin in these membranes and these experiments are still in progress.

This study shows that it is possible to make valid assumptions about the relationship between agonist binding and response by a direct comparison of agonist dose-response curves with their binding curves. This assertion is considerably strengthened by the fact that the efficacy of pilocarpine relative to that of carbachol in striatal membranes (0.03) compares very favorably with that found by Furchgott and Bursztyn (11) from studies on contraction of rat stomach muscle strips, for which they obtained a value of 0.05.

However, the relative efficacies of the agonists studied here are clearly different in the striatal and myocardial preparations (Table 3). As relative efficacy is a receptor-specific parameter independent of tissue and response (10, 11) this is good evidence of a real conformational difference between the receptors and/or receptor-effector molecule complexes in the two preparations. As efficacy is presumed to reflect the interaction between the agonist-receptor complex and the G protein (see above), it is not possible to determine whether the conformational difference is between uncoupled receptor or between the *R–G* complexes from differences in relative efficacy alone. However, there is a significant difference in the apparent affinity of carbachol for receptors in the striatal and myocardial preparations, in the presence of guanine nucleotide (Table 1). As this presumably reflects differences in carbachol affinity for uncoupled receptors in the two preparations, it suggests that the conformations of the receptor molecules themselves are different and is consistent with evidence that the primary structures

of muscarinic receptors in forebrain and heart seem to differ (6).

Acknowledgments

The authors wish to thank Merck Sharp and Dohme Neuroscience Research Centre (UK) for financial support, Dr. D. R. Hill for help and advice, and Jenny Bell for manuscript preparation.

References

1. Murad, F., Y.-M. Chi, T. W. Rall, and E. W. Sutherland. The effect of catecholamines and choline esters on the formation of adenosine 3′,5′-phosphate by preparations from cardiac muscle and liver. *J. Biol. Chem.* **237**:1233–1238 (1962).
2. Olianas, M. C., P. Onali, N. H. Neff, and E. Costa. Adenylate cyclase activity of synaptic membranes from rat striatum: inhibition by muscarinic receptor agonists. *Mol. Pharmacol.* **23**:393–398 (1983).
3. Ehlert, F. J. The relationship between muscarinic receptor occupancy and adenylate cyclase inhibition in the rabbit myocardium. *Mol. Pharmacol.* **28**:410–421 (1985).
4. Hammer, R., C. P. Berrie, N. J. M. Birdsall, A. S. V. Burgen, and E. C. Hulme. Pirenzepine distinguishes between different subclasses of muscarinic receptors. *Nature* (*Lond.*) **283**:90–92 (1980).
5. Hammer, R., E. Giraldo, G. B. Schiavi, E. Monferini, and H. Ladinsky. Binding profile of a novel cardioselective muscarine receptor antagonist, AFDX 116, to membranes of peripheral tissues and brain in the rat. *Life Sci.* **38**:1653–1662 (1986).
6. Kubo, T., A. Maeda, K. Sugimoto, I. Akiba, A. Mikami, H. Takahashi, T. Haga, K. Haga, A. Ichiyama, K. Kangawa, H. Matsuo, T. Hirose, and S. Numa. Primary structure of porcine cardiac muscarinic acetylcholine receptor deduced from the cDNA sequences. *FEBS Lett.* **209**:367–372 (1986).
7. Hammer, R., and A. Giachetti. Muscarinic receptor subtypes: M_1 and M_2 biochemical and functional characterization. *Life Sci.* **31**:2991–2998
8. Eglen, R. M., B. A. Kenny, A. D. Michel, and R. L. Whiting. Muscarinic activity of McN-A-343 and its value in muscarinic receptor classification. *Br. J. Pharmacol.* **90**:693–700 (1987).
9. Stephenson, R. P. A modification of receptor theory. *Br. J. Pharmacol.* **11**: 379–393 (1956).
10. Jenkinson, D. H. Partial agonists in receptor classification, in *Medicinal Chemistry VI* (A. Simkin, ed.). Cotswold Press, Oxford, 373–383 (1979).
11. Furchgott, R. F., and P. Bursztyn. Comparison of dissociation constants and of relative efficacies of selected agonists acting on parasympathetic receptors. *Ann. N. Y. Acad. Sci.* **144**:882–899 (1967).
12. Gill, E. W., and H. P. Rang. An alkylating derivative of benzilylcholine with specific and long-lasting parasympathetic activity. *Mol. Pharmacol.* **2**:284–297 (1966).
13. Brown, B. L., J. D. M. Albano, R. P. Ekins, A. M. Sgherzi, and W. Tampion. A simple and sensitive saturation assay method for the measurement of adenosine 3′:5′-cyclic monophosphate. *Biochem. J.* **121**: 561–562 (1971).
14. Salomon, Y., D. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548 (1974).
15. De Lean, A., P. J. Munson, and D. Rodbard. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay and physiological dose-response curves. *Am. J. Physiol.* **235**:E97–E102 (1978).
16. Dickinson, K., A. Richardson, and S. R. Nahorski. Homogeneity of *beta*$_2$-adrenoceptors on rat erythrocytes and reticulocytes: a comparison with heterogeneous rat lung *beta*-adrenoceptors. *Mol. Pharmacol.* **19**:194–204 (1981).
17. Birdsall, N. J. M., A. S. V. Burgen, and E. C. Hulme. The binding of agonists to brain muscarinic receptors. *Mol. Pharmacol.* **14**:723–736 (1978).
18. Evans, T., J. R. Hepler, S. B. Masters, J. H. Brown, and T. K. Harden. Guanine nucleotide regulation of agonist binding to muscarinic cholinergic receptors: relation to efficacy of agonists for stimulation of phosphoinositide breakdown and Ca^{2+} mobilization. *Biochem. J.* **232**:751–757 (1985).
19. Aronstam, R. S., and L. M. Greenbaum. Guanine nucleotide sensitivity of muscarinic acetylcholine receptors from rat brainstem is eliminated by endogenous proteolytic activity. *Neurosci. Lett.* **47**:131–137 (1984).
20. Christophe, J., P. De Neef, P. Robberecht, and M. Waelbroeck. Propylbenzilylcholine mustard is selective for rat heart muscarinic receptors having a low affinity for agonists. *Br. J. Pharmacol.* **88**:63–70 (1986).
21. Furchgott, R. F. The use of β-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor-agonist complexes. *Adv. Drug Res.* **3**:21–55 (1986).
22. Colquhoun, D. Affinity, efficacy and receptor classification: is the classical theory still useful, in *Perspectives on Receptor Classification* (J. W. Black, D. H. Jenkinson, and V. P. Gerskowitch, eds.). Alan R. Liss, New York 103–114 (1987).
23. Gilman, A. G. G-proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56**:615–649 (1987).
24. Ehlert, F. J. The relationship between muscarinic receptor occupancy and adenylate cyclase inhibition in the rabbit myocardium. *Mol. Pharmacol.* **28**:410–421 (1985).
25. Berrie, C. P., N. J. M. Birdsall, E. C. Hulme, M. Keen, and J. M. Stockton. Solubilisation and characterisation of guanine nucleotide-sensitive muscarinic agonist binding sites from rat myocardium. *Br. J. Pharmacol.* **82**:853–861 (1984).
26. Rooney, T. A., and S. R. Nahorski. Regional charcterization of agonist and depolarization-induced phosphoinositide hydrolysis in rat brain. *J. Pharmacol. Exp. Ther.* **239**:873–879 (1986).
27. Brown, J. H., D. Goldstein, and S. B. Masters. The putative M_1 muscarinic receptor does not regulate phosphoinositide hydrolysis: studies with pirenzepine and McN-A343 in chick heart and astrocytoma cells. *Mol. Pharmacol.* **27**:525–531 (1985).
28. Breitwieser, G. E., and G. Szabo. Uncoupling of cardiac muscarinic and β-adrenergic receptors from ion channels by a guanine nucleotide analogue. *Nature* (*Lond.*) **317**:538–540 (1985).
29. Pfaffinger, P. J., J. M. Martin, D. D. Hunter, N. M. Nathanson, and B. Hille. GTP-binding proteins couple cardiac muscarinic receptors to a K channel. *Nature* (*Lond.*) **317**:536–538 (1985).
30. Kelly, E., T. A. Rooney, and S. R. Nahorski. Pertussis toxin separates two muscarinic receptor-effector mechanisms in the striatum. *Eur. J. Pharmacol.* **119**:129–130 (1985).
31. Watson, M., H. I. Yamamura, and W. R. Roeske. A unique regulatory profile and regional distribution of ^{3}H-pirenzepine binding in the rat provide evidence for distinct M_1 and M_2 receptor subtypes. *Life Sci.* **32**:3001–3011 (1983).
32. Black, J. W., P. Leff, N. P. Shankley, and J. Wood. An operational model of pharmacological agonism: the effect of $E/[A]$ curve shape on agonist dissociation constant estimation. *Br. J. Pharmacol.* **84**:561–571 (1985).
33. Ehlert, F. J. Coupling of muscarinic receptors to adenylate cyclase in the rabbit myocardium: effects of receptor inactivation. *J. Pharmacol. Exp. Ther.* **240**:23–30 (1987).
34. Hazeki, O., and M. Ui. Modification by islet-activating protein of receptor-mediated regulation of cAMP accumulation in isolated rat heart cells. *J. Biol. Chem.* **256**:2856–2862 (1981).

Send reprint requests to: Stefan R. Nahorski, Department of Pharmacology and Therapeutics, University of Leicester, P.O. Box 138, Medical Science Building, University Rd., Leicester LE1 9HN, UK.