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The Relationship between Muscarinic Receptor Occupancy and Adenylate Cyclase Inhibition in the Rabbit Myocardium

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SUMMARY

The muscarinic receptor-binding properties of a series of muscarinic drugs were compared with their effects on adenylate cyclase in membranes of the rabbit myocardium. When measured by competitive inhibition of [^{3}H]-*N*-methylscopolamine binding, the competition curves of the various agonists were adequately described by the ternary complex model. This model assumes that the receptor can bind reversibly with a guanine nucleotide binding protein in the membrane and that the affinity of the agonist for the receptor-guanine nucleotide-binding protein complex is higher than that for the free receptor. A satisfactory fit of the ternary complex model to the data could only be achieved assuming that very little receptor is precoupled with the guanine nucleotide-binding protein in the absence of agonist. There was good agreement between the efficacy of each agonist as measured by inhibition of adenylate cyclase and the estimate of the positive cooperativity between the binding of the agonist receptor complex and the guanine nucleotide-binding protein. Guanosine 5′-triphosphate (0.1 mM) had no significant effect on the binding of [^{3}H]*N*-methylscopolamine but caused an increase in the concentration of the various agonists required for half-maximal receptor occupancy. There was good correlation between efficacy as measured by inhibition of adenylate cyclase and the influence of guanosine 5′-triphosphate on binding properties.

INTRODUCTION

Stimulation of muscarinic receptors causes a variety of responses in intact tissues. Although many of the events between receptor activation and cellular response are unknown, some biochemical consequences of muscarinic stimulation have been identified which are probably important links in the chain of events of the muscarinic response. One such event is the inhibition of adenylate cyclase. Muscarinic agonists inhibit the accumulation of cyclic AMP in a variety of whole and broken cell preparations (1–5). In intact thyroid cells, the effect is mediated by a phosphodiesterase (4), whereas in homogenates of the myocardium and striatum, muscarinic agonists inhibit adenylate cyclase (1, 2, 5).

What has emerged as a general theory (6) for the coupling of various membrane receptors to adenylate cyclase and other effectors can also be applied to the muscarinic receptor; namely, that a guanine nucleotide-binding protein is involved in the coupling of the muscarinic receptor to adenylate cyclase. Strong evidence supports this theory. In rat striatum, for example, the muscarinic inhibition of adenylate cyclase has been associated with the stimulation of a high affinity GTPase (7). The islet-activating protein, pertussis toxin, which catalyzes the ADP ribosylation of the inhibitory guanine nucleotide-binding protein (N_i) (8) and thereby prevents various receptors from inhibiting adenylate cyclase, has been shown to uncouple muscarinic receptors from adenylate cyclase (9). Finally, guanine nucleotides modify the binding properties of muscarinic receptors (10–12), and this behavior is particularly demonstrable in the heart where GTP (0.1 mM) has been shown to cause an increase of approximately 20-fold in the concentration of carbachol necessary for half-maximal receptor occupancy (10). Thus, the heart is a very suitable tissue for investigating the relationship between the binding properties of muscarinic agonists and the inhibition of adenylate cyclase.

It has become apparent that both the affinity and efficacy of muscarinic drugs influence the way in which they bind with muscarinic receptors. For example, the binding isotherms of most muscarinic antagonists, like atropine, are consistent with a simple one-site model, whereas the binding isotherms of efficacious agonists, like oxotremorine-M and carbachol, are complex and have Hill coefficients less than 1 (13). This difference between the binding of agonists and antagonists has been rationalized on the basis of multiple states or classes of binding sites having different affinities for agonists and

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equal affinity for most antagonists (13). A plausible model for cardiac muscarinic receptors is that the receptor interacts with an intrinsic guanine nucleotide-binding protein and that the occupation of the receptor by an agonist promotes the formation of a ternary complex consisting of the agonist, receptor, and guanine nucleotide-binding protein. This model has been applied to numerous receptors which affect adenylate cyclase including the muscarinic receptor. The precise mathematical consequences of a model where the active drug-receptor complex promotes the coupling with a membrane protein or effector have been described by Jacobs and Cuatrecasas (14) within the context of their "mobile receptor hypothesis" and more recently by Wreggett and De Lean (15) to describe the consequences of the "ternary complex model." Although numerous investigators have interpreted agonist-binding isotherms and the effects of GTP on them within the context of the ternary complex model, relatively few attempts have been made to estimate the fundamental parameters of the model. More commonly, binding data have been analyzed in terms of a model of two independent sites, and from the results of such an analysis, intuitive conclusions about the relative abundances of receptor and guanine nucleotide-binding protein, their affinity for each other, and the cooperative interactions between drugs and the guanine nucleotide-binding protein have been deduced. In the present report, I have derived an equation describing the ternary complex model of De Lean *et al.* (16) and have analyzed the binding data of a series of muscarinic drugs including full agonists, partial agonists, and antagonists (see Fig. 1) according to the model. The results of this analysis showed that the theoretical estimates of the affinity and efficacy of a series of muscarinic drugs agree very well with those measured directly by inhibition of adenylate cyclase.

MATERIALS AND METHODS

Tissue preparation. Male New Zealand White rabbits (1.4–1.8 kg) were killed, and their hearts were removed rapidly, perfused through the aorta with ice-cold saline, and minced with scissors. The minced tissue was homogenized at 0° with the Polytron in a buffer containing 0.25 M sucrose and 0.02 M Na/HEPES[1] (pH 7.5) and poured through three layers of cheesecloth. The final tissue concentration was 10% (w/v). This homogenate was centrifuged at 30,000 × *g* for 10 min, and the resultant pellet was resuspended with a Potter-Elvehjem glass homogenizer and Teflon pestle in two different ways, depending upon whether the tissue was used in the adenylate cyclase assay or in the muscarinic receptor-binding assay. For the cyclase assay, the pellet was resuspended to a final concentration of 40 mg of tissue (original wet weight)/ml of buffer containing 0.25 M sucrose, 0.02 M Na/HEPES (pH 7.5) and 2.0 mM DTT. For the binding assay, the pellets were resuspended to a final concentration of 8.0 mg of tissue (original wet weight)/ml of buffer containing 0.035 M Na/HEPES (pH 7.5) and 1.0 mM DTT.

Adenylate cyclase assay. Myocardial homogenate (0.05 ml) was incubated at 30° for 10 min in a final volume of 0.2 ml containing 0.035 M Na/HEPES (pH 7.5), 0.063 M sucrose, 0.5 mM DTT, 0.5–1.0 μCi of [α^{32}P]ATP, 0.5 mM ATP, 1.0 mM cyclic AMP, 5.0 mM phosphocreatine, 30 units/ml of creatine phosphokinase, 0.1 M NaCl, 5.0 mM $MgCl_2$, 1.0 mM isobutylmethylxanthine, 0.5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid and various muscarinic drugs as described in Results. The reaction was started by adding tissue and was stopped by adding 0.1 ml of a solution of 40 mM ATP, 1.4 mM cyclic AMP, and 0.1 mM sodium dodecyl sulfate. An aliquot (0.8 ml) of water containing 10,000 cpm of [^{3}H]cyclic AMP was added subsequently, and [^{32}P]cyclic AMP was determined using the method of Salomon *et al.* (17). The average recovery of cyclic AMP was about 80%.

Binding assay. Myocardial homogenate (1.0 ml) was incubated at 30° for 10 min in a final volume of 2 ml containing [^{3}H]NMS (84.4 Ci/mmol; New England Nuclear, Boston, MA), 0.035 M Na/HEPES (pH 7.5), 0.1 M NaCl, 0.5 mM DTT, 5.0 mM $MgCl_2$, and various muscarinic drugs as described in Results. Tissue-bound [^{3}H]NMS was trapped subsequently by rapid vacuum filtration of the incubation mixture over

[1] The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; NMS, *N*-methylscopolamine; RSS, residual sum of squared deviations.

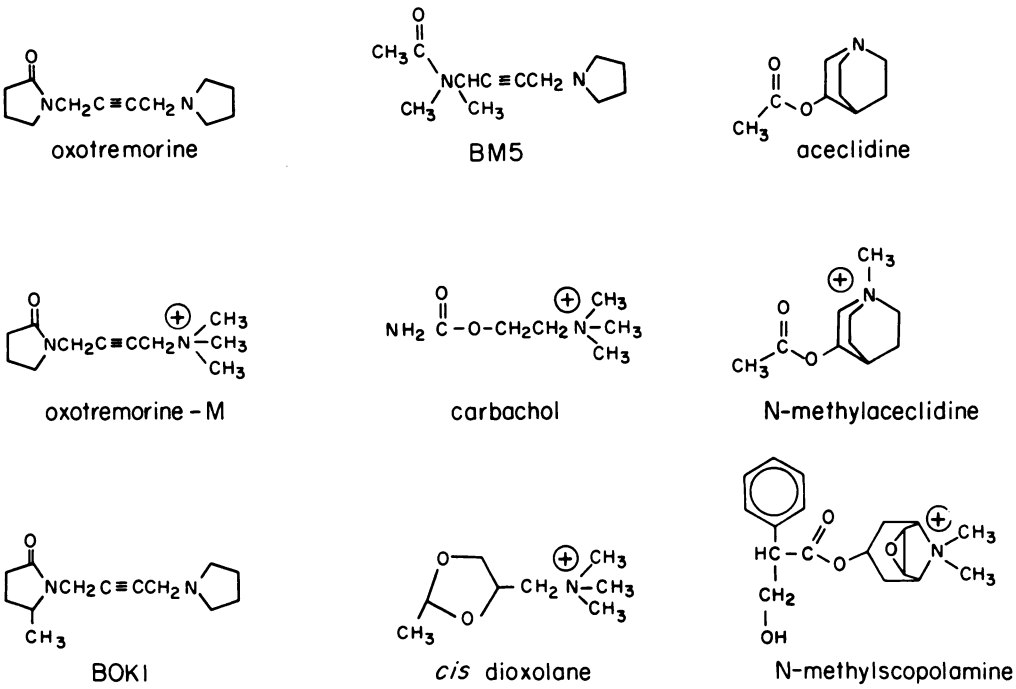

FIG. 1. *Structures of the cholinergic analogs used in this study.*

Whatman glass fiber filters (GF/B). The filters were rinsed with three aliquots (3 ml each) of ice-cold saline. Nonspecific binding was estimated by carrying out incubations in the presence of 10 μM atropine.

Analysis of binding data. Since there is a large difference in the pharmacological activity of the enantiomers of NMS (18), the binding data for [^{3}H]NMS were calculated assuming that both enantiomers of racemic [^{3}H]NMS bound nonspecifically to the same extent but that only the (−)-enantiomer contributed to specific binding. The muscarinic receptor-binding properties of a series of drugs were investigated by measuring their ability to inhibit specific [^{3}H]-(−)-NMS binding competitively. The data were analyzed by nonlinear regression analysis according to Eq. A14 in the Appendix which represents an explicit solution to the ternary complex model in a form which lends itself to analysis of drug/^{3}H-ligand competition curves.

The ternary complex model assumes that the receptor (R) and the guanine nucleotide-binding protein (N) diffuse within the plane of the membrane and bind with each other as predicted by the affinity constant (K_2). The drug (D) has an allosteric effect on this equilibrium by binding to the recognition site on the receptor as shown below:

$$\begin{array}{ccc} & \overset{K_1}{\rightleftharpoons} & \\ D + R + N & & DR + N \\ K_2 \uparrow\downarrow & & \uparrow\downarrow \alpha K_2 \\ D + RN & \underset{\alpha K_1}{\rightleftharpoons} & DRN \end{array}$$

In this scheme, K_1 is the affinity constant of the drug for the receptor, DR is the drug-receptor complex, and RN is the receptor-guanine nucleotide-binding protein complex. The law of conservation of the standard free energy as it applies to equilibria between a protein and two ligands requires that the ligands have reciprocal effects on the binding of each other (19) so that the effect that N produces on the binding of D is equivalent to the effect that D produces on the binding of N. This reciprocal cooperativity is denoted by α. Thus, αK_1 represents the affinity constant for the binding of D to RN, and αK_2 represents the affinity constant for the binding of N to DR, and DRN is the resultant ternary complex. As shown in the Appendix, it is convenient to introduce the parameter β which denotes the ratio of the total concentrations of guanine nucleotide-binding site and receptor (N_T/R_T).

The concentration of nonlabeled drug required for half-maximal receptor occupancy (D_{50}) was calculated from the concentration of drug which caused half-maximal inhibition of specific [^{3}H]-(−)-NMS binding (IC_{50}) using the following equation:

$$D_{50} = IC_{50}/(1 + [[^3H]\text{-}(-)\text{-}NMS]K_{NMS}) \quad (1)$$

In this equation, K_{NMS} is equal to the affinity constant of [^{3}H]-(−)-NMS.

RESULTS

Muscarinic inhibition of adenylate cyclase. In the presence of increasing concentrations (0.1–100 μM) of GTP, adenylate cyclase activity increased from a basal level of 5.75 ± 0.05 to a plateau value of 7.90 ± 0.06 pmol min^{-1} mg^{-1} tissue at 100 μM GTP. The concentration of GTP causing half-maximal stimulation of the enzyme was 0.22 μM. GTP-stimulated adenylate cyclase activity was linear with tissue concentration over the range of 5–20 mg of tissue (original wet weight)/ml of incubation medium, and the rate of formation of cyclic AMP was constant over time from 0–16 min. Oxotremorine-M (100 μM) almost fully prevented the stimulatory effect of GTP on adenylate cyclase activity. Adenylate cyclase activity in the presence of GTP (100 μM) and oxotremorine-M (100 μM) was only 68–72% of that measured in the presence of GTP alone. Atropine (1.0 μM) completely blocked the inhibition of adenylate cyclase activity caused by oxotremorine-M (100 μM).

The effects of various concentrations of cholinergic analogues on adenylate cyclase activity assayed in the presence of GTP (100 μM) are shown in Figs. 2 and 3. Among the compounds tested, carbachol, *cis*-dioxolane, oxotremorine, and oxotremorine-M all caused a similar maximal inhibition (27–28%) of adenylate cyclase activity, whereas the remainder of the compounds had effects which reached a plateau at lower levels of enzyme inhibition (4–25%). Table 1 lists the maximal degree of enzyme inhibition caused by each compound together with the concentration of drug (K_i) causing half of its maximal effect. The effects of carbachol on adenylate cyclase activity measured in the absence and presence of NMS (10 nM) and the oxotremorine analogues BOK1 (3 μM) and BM5 (3 μM) are shown in Fig. 3. Both NMS and BOK1 behaved as competitive antagonists and

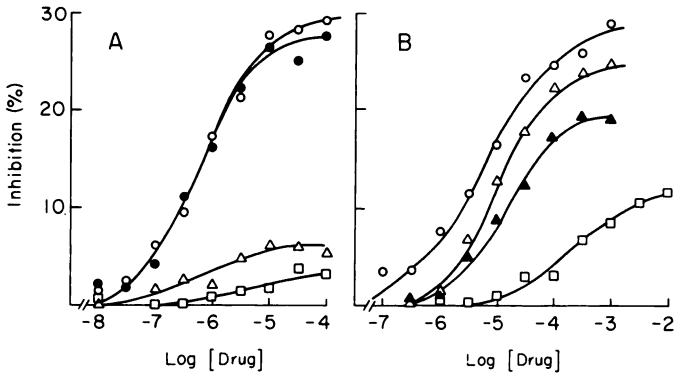

FIG. 2. *Inhibition of adenylate cyclase activity in the rabbit myocardium by various cholinergic drugs*

Each point represents the mean value of four experiments, each done in quadruplicate. The average standard error of the mean was 2.01% inhibition. Adenylate cyclase activity was measured in the absence and presence of the indicated concentrations of the following drugs: A, oxotremorine-M (○), oxotremorine (●), BM5 (△), and BOK1 (□); B, *cis*-dioxolane (○), (+)-aceclidine (△), (−)-aceclidine (▲), and *N*-methylaceclidine (□).

TABLE 1

Inhibition of myocardial adenylate cyclase by various muscarinic agents

Compound	pK_i[a]	Maximum inhibition (%)[b]	Efficacy[c]
Oxotremorine-M	6.2	28.2	3.6
Carbachol	4.6	28.4	2.3
cis-Dioxolane	5.5	27.9	2.7
Oxotremorine	6.2	26.8	1.2
(+)-Aceclidine	4.9	24.0	0.85
(−)-Aceclidine	4.8	18.4	0.65
N-Methylaceclidine	4.0	11.5	0.41
BM5	6.0	5.2	0.20
BOK1	5.5	3.2	0.11

[a] Negative logarithm of the concentration of drug causing half of the maximal inhibition of adenylate cyclase activity observed at the highest concentration of the drug.

[b] Maximum inhibition of adenylate cyclase activity observed at the highest concentration of the drug.

[c] Efficacy was calculated according to Eqs. 4 and 5 as described in Results.

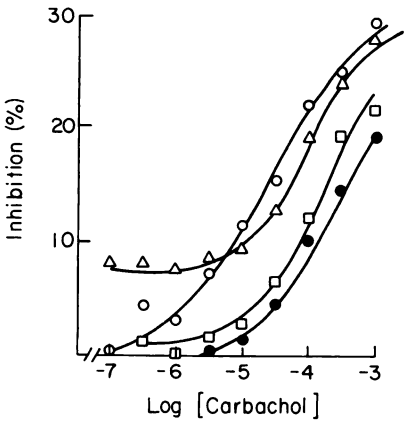

FIG. 3. *Inhibition of adenylate cyclase activity in the rabbit myocardium by carbachol in the absence (○) and presence of 10 nM NMS (●), 3 μM BM5 (△), and 3 μM BOK1 (□)*

Each *point* represents the mean value of four experiments, each done in quadruplicate. The average standard error of the mean was 1.50% inhibition.

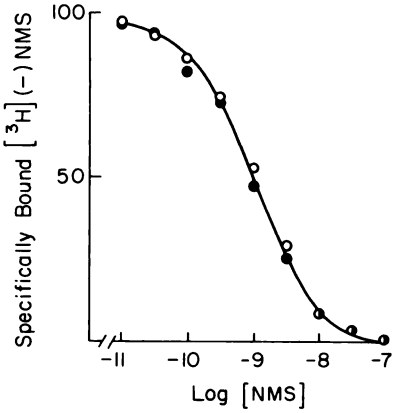

FIG. 4. *Competitive inhibition of specific [³H]-(−)-NMS binding to the rabbit myocardium by NMS in the absence (○) and present (●) of GTP (0.1 mM)*

Each *point* represents the mean binding value of five experiments, each done in triplicate. The average standard error of the mean was 2.07%. The concentration of [³H]NMS was 0.5 nM. The theoretical curve represents the least squares fit to the data.

shifted the carbachol dose response curve to the right 16- and 8-fold, respectively. The effects of BM5 on the carbachol dose response curve resembled those of a partial agonist. In the absence of carbachol, BM5 (3 μM) caused some basal level of enzyme inhibition (5%), and adding increasing concentrations of carbachol to the assay caused a further inhibition of the enzyme to the maximal level. The concentration of carbachol causing half-maximal inhibition over the basal level increased 4-fold in the presence of BM5. The K_I values of NMS, BOK1, and BM5 for antagonizing the effects of carbachol were calculated from the competitive inhibition relationship:

$$DR - 1 = [I]/K_I \qquad (2)$$

in which $[I]$ represents the molar concentration of the inhibitor, K_I represents the dissociation constant of the inhibitor, and DR represents the ratio of concentrations of carbachol causing half-maximal inhibition of adenylate cyclase activity measured in the presence and absence of the inhibitor, respectively. These calculations yielded K_I values of 0.67 nM, 0.43 μM, and 1.0 μM for NMS, BOK1, and BM5, respectively.

Muscarinic receptor binding properties. When measured at a ³H-ligand concentration of 0.5 nM, specific [³H]-(−)-NMS binding reached equilibrium within 10 min at 30°. The mean specific binding value ± the standard error for [³H]-(−)-NMS reached a near-maximum at 4 min (32.2 ± 2.1 fmol) and did not increase much above this plateau at 10 (35.8 ± 1.7 fmol), 15 (34.4 ± 1.5 fmol), and 20 min (29.0 ± 2.0) of incubation. Previous studies have shown that [³H]NMS binding reaches equilibrium within 10 min in the myocardium (20) and that the percentage of inhibition of [³H]NMS binding by agonists is constant over time from 2–10 min (10).

The effects of GTP on the binding of [³H]-(−)-NMS were investigated by measuring the competitive inhibition of [³H]-(−)-NMS binding by nonlabeled NMS in the presence and absence of GTP (0.1 mM). It can be seen in Fig. 4 that GTP had no significant effect on the NMS/[³H]-(−)-NMS competition curve. Nonlinear regression analysis of the competition curve showed that the data were consistent with a one-site model, with the affinity constant of (−)-NMS being 4.5×10^9 M^{-1}. There was no significant reduction in residual error when the data were fitted to a two-independent-site model or to the ternary complex model. The good agreement between the data and a simple one-site model can be seen by the theoretical curve in Fig. 4 which represents the least squares fit of the data to Eq. A14 assuming no cooperativity ($\alpha = \gamma = 1$) and $K_1 = K_3$.

The receptor-binding properties of a series of muscarinic agonists were investigated by measuring the competitive inhibition of specific [³H]-(−)-NMS binding in the presence and absence of GTP (0.1 mM) as shown in Figs. 5 and 6. GTP caused an increase in the concentration of each drug required for half-maximal receptor occupancy (D_{50}). The largest effects of GTP were noted in competition experiments with oxotremorine-M, carbachol, and *cis*-dioxolane. Their D_{50} values increased 63- to 141-fold in the presence of GTP. Intermediate increases (18- to 40-fold) in the D_{50} values of oxotremorine, and the enantiomers of aceclidine were noted in the presence of GTP, whereas only small increases (3.5- to 6-fold) were noted in the D_{50} values of *N*-methylaceclidine, BOK1, and BM5 in the presence of GTP. Table 2 summarizes the effects of GTP on the D_{50} values of the various cholinergic analogues shown in Figs. 4–6.

All of the agonist competition curves measured in the absence of GTP were inconsistent with a simple one-site model. A substantial inhibition of [³H]-(−)-NMS binding occurred over a relatively broad range of concentrations of the nonlabeled agonists, and consequently the slopes of the various agonist/[³H]-(−)-NMS competition curves were not as steep as that expected for a simple one-site model. Nonlinear regression analysis of the data according to Eq. A14 showed that the data were consistent with the ternary complex model. However, it was not possible to determine all four parameters (K_1, K_2, α, and β) in the ternary complex model by regression analysis of the competition curves, because it is necessary to collect data

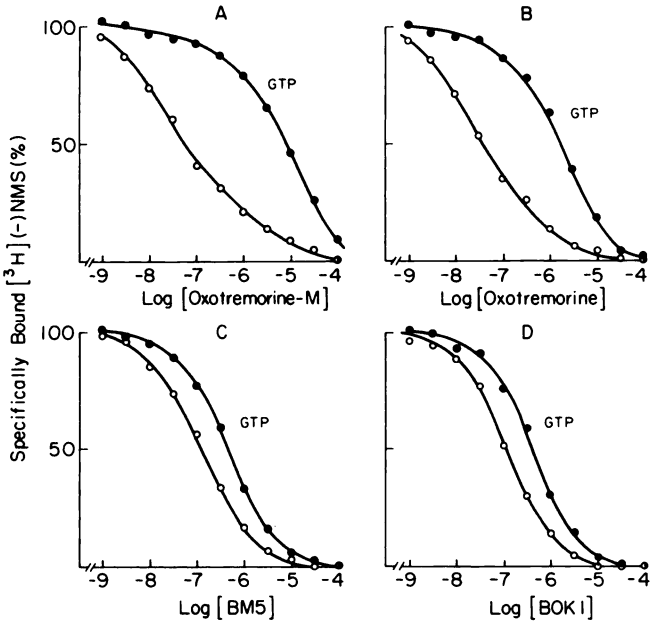

FIG. 5. *Competitive inhibition of specific [³H]-(−)-NMS binding to the rabbit myocardium by oxotremorine-M (A), oxotremorine (B), BM5 (C), and BOK1 (D)*

Assays were run in the absence (O) and presence (●) of GTP (0.1 mM). Each *point* represents the mean binding value of five experiments, each done in triplicate. The average standard error of the mean was 2.21%. The concentration of [³H]NMS was 0.5 nM. The theoretical curves for the competition data measured in the absence of GTP represent the least squares fit to the data according to Eq. A14.

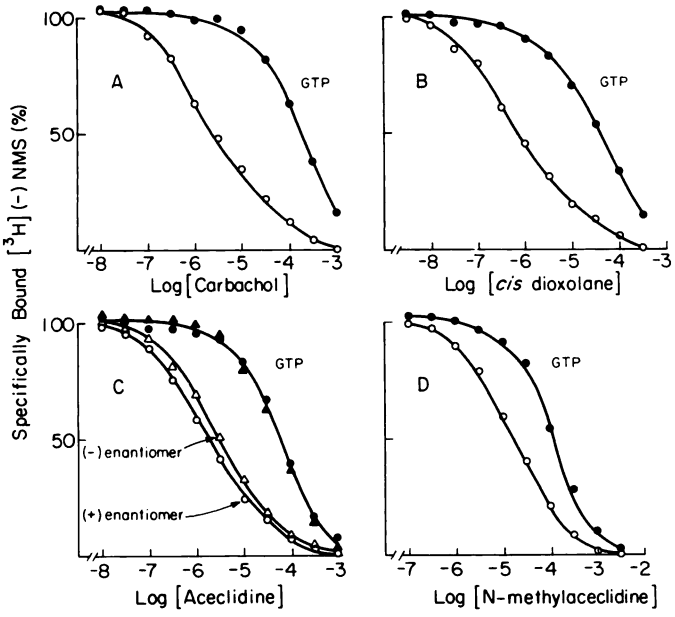

FIG. 6. *Competitive inhibition of specific [³H]-(−)-NMS binding to the rabbit myocardium by carbachol (A), cis-dioxolane (B), (+)- and (−)-aceclidine (C), and N-methylaceclidine (D)*

Other details of the assay are the same as those described in the legend to Fig. 5. The average standard error of the mean was 1.96%.

at various values of β (N_T/R_T) as well as at various concentrations of agonist in order to define the system fully. Since the ratio N_T/R_T is fixed at a value inherent to cardiac membranes, it was not possible to estimate

TABLE 2

Effect of GTP (0.1 mM) on the binding of various compounds to muscarinic receptors in the myocardium

Compound	pD_{50}[a]	$pD_{50\text{-GTP}}$[b]	$\frac{D_{50\text{-GTP}}}{D_{50}}$
Oxotremorine-M	7.6	5.4	141
Carbachol	5.8	4.0	56
cis-Dioxolane	6.6	4.8	63
Oxotremorine	7.6	6.0	40
(+)-Aceclidine	6.0	4.4	36
(−)-Aceclidine	5.9	4.6	18
N-Methylaceclidine	5.0	4.3	5.6
BM5	7.3	6.7	4.0
BOK1	7.3	6.7	3.5
(−)-NMS	9.7	9.7	1.0

[a] Negative logarithm of the concentration of drug required for half-maximal receptor occupancy.

[b] Negative logarithm of the concentration of drug required for half-maximal receptor occupancy in the presence of GTP.

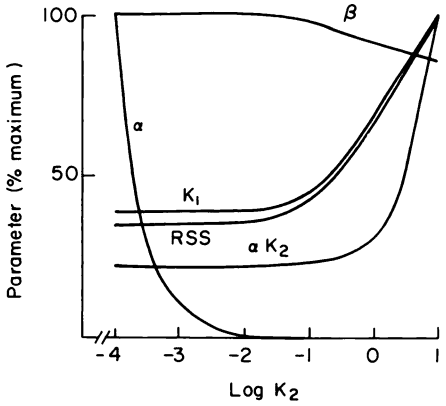

FIG. 7. *Summary of nonlinear regression analysis of the oxotremorine-M/[³H]-(−)-NMS competition data according to the ternary complex model*

The oxotremorine-M/[³H]-(−)-NMS competition data shown in Fig. 5A were analyzed by nonlinear regression analysis according to Eq. A14. In these calculations, the value of K_2 was fixed at various values between 10^{-4} and 10, and the estimates of the other parameters which minimized the sum of the squared deviations between the observed and predicted binding values (*RSS*) were determined and are shown by the indicated curves. The value of each parameter is scaled to its maximum value attained over the range of K_2 values shown above.

the affinity constant (K_2) of the *NR* complex. Fig. 7 summarizes the results of regression analysis of the control oxotremorine-M/[³H]-(−)-NMS competition data according to Eq. A14. In these calculations, K_2 was fixed at various values between 10^{-4} and 10 and the estimates of the other parameters which minimized the residual sum of squared deviations (RSS) between the data points and the theoretical binding values were calculated by nonlinear regression analysis. It can be seen that as the value of K_2 decreased to 10^{-2}, the value of RSS approached a minimum and remained constant over the range of 10^{-2}–10^{-4}. Similarly, when the value of K_2 was equal to or less than 10^{-2}, the parameter estimates of β and K_1 plateaued at constant values. In contrast, when the value of K_2 was equal to or less than 10^{-2}, the estimate of α increased proportionately with the reciprocal of K_2 so that the product (αK_2) of the two estimates

remained constant. Fixing K_2 to values in the range of 10^{-8}–10^{-4} yielded parameter estimates for K_1, β, and αK_2 which were equivalent to those determined at $K_2 = 10^{-4}$ (data not shown). Thus, it was possible to determine the best estimates of K_1, β, and the product αK_2 by fixing K_2 to an arbitrarily low value (10^{-4}) and determining the parameter estimates which minimized RSS. Regression analysis of the competition curves of the various muscarinic agonists yielded results similar to those shown for oxotremorine-M in Fig. 7, with RSS being at a constant minimum over the range (10^{-8}–10^{-2}) of K_2 values and the estimates of the parameters K_1, β, and αK_2 being constant over the same range. Preliminary regression analysis of the various agonist/[^{3}H]-(−)-NMS competition curves showed that the estimates of β remained relatively constant and independent of the drug. Since errors in the estimates of parameters are correlated, it is best to do regression analysis sharing parameters which are constant among the various sets of data. It was thought that the best estimate of β would be obtained from those compounds (oxotremorine-M, oxotremorine, *cis*-dioxolane, carbachol, and (+)-aceclidine) having the largest value of αK_2. The mean estimate of β ± the standard deviation for these compounds was 0.81 ± 0.04. Table 3 summarizes the results of regression analysis of the competition data assuming a constant value (0.81) of β and an arbitrarily low value (10^{-4}) of K_2. Analysis of variance showed no significant increase in residual error when the value of β was held constant among the various curves ($F_{9,71} = 1.327$; $p = 0.24$).

Using the parameter estimates shown in Table 3 and Eqs. A15 and A16, it is possible to calculate the relative amounts of drug receptor complex (*DR*) and ternary complex (*DRN*) at various concentrations of the cholinergic analogues as described in the Appendix. Fig. 8 shows the results of such calculations for oxotremorine-M and *N*-methylaceclidine. The *solid curves* in Fig. 8, A and B, represent the best fit to the data assuming the ternary complex model, and the *dashed line* in Fig. 8A represents the best fit assuming two independent binding sites. Both the two-independent-site model and the ternary complex model yielded theoretical binding curves for the *N*-methylaceclidine/[^{3}H]-(−)-NMS competition data which were nearly superimposable; hence, only one curve is shown in Fig. 8B. Analysis of variance showed that the variance estimate based on deviations between the mean data points and the predicted binding values of both models was not significantly greater than that

TABLE 3

Binding parameters of muscarinic agonists

Compound	Log (K_1)	αK_2
Oxotremorine-M	5.5	381
Carbachol	4.1	187
cis-Dioxolane	4.8	157
Oxotremorine	6.2	95
(+)-Aceclidine	4.7	66
(−)-Aceclidine	4.6	46
N-Methylaceclidine	4.1	22
BM5	6.3	17
BOK1	6.6	8.6

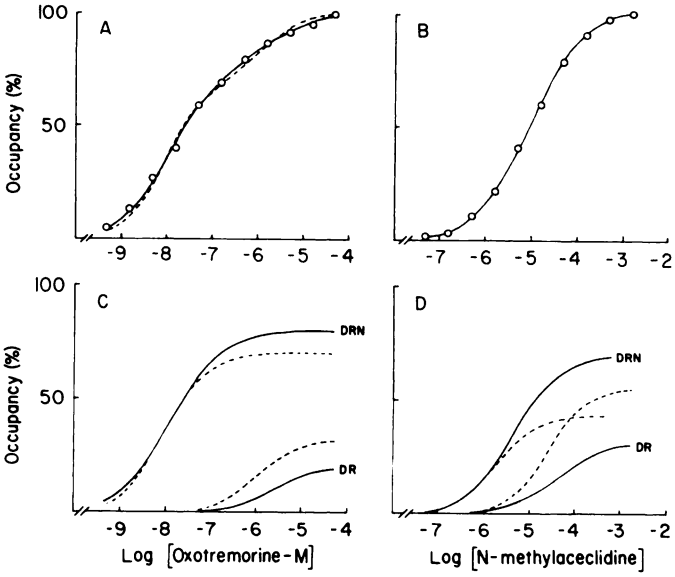

FIG. 8. *Analysis of the oxotremorine-M (A, C) and N-methylaceclidine (B, D) binding data according to the ternary complex model (solid lines) and the two-independent-site model (dashed lines)*

Occupancy was calculated as the percentage of specifically bound [^{3}H]-(−)-NMS displaced by each drug. The occupancy curves have been corrected for the competitive shift caused by [^{3}H]-(−)-NMS. The theoretical curves in A and B represent the best fit to the data. Both the ternary complex model and the two-site model gave similar fits for the *N*-methylaceclidine data; hence, only one curve is shown. The curves in C and D represent the two components of each model.

estimated by replicate measurements of binding. Consequently, both the ternary complex model and the two-independent-site model provided a reasonable fit to the data. In Fig. 8, C and D, the *solid lines* show the relative contributions of *DRN* and *DR* to receptor occupancy as individual components, and the *dashed lines* show the high and low affinity components of the two-independent-site model. It can be seen that the high and low affinity components of the two-independent-site model provide rough estimates of the proportion of bound drug in the form of ternary complex (*DRN*) and drug receptor complex (*DR*). The relative amounts of *DRN* and *DR* were determined for all of the compounds using the parameter estimates in Table 3 and Eqs. A15 and A16. The results of these calculations are summarized in Table 4 which lists the concentration of drug resulting in half-maximal formation of *DRN* and *DR* and the theoretical ratio of DRN/R_T at 100% receptor occupancy.

Efficacy determinations. The relationship between receptor occupancy and adenylate cyclase inhibition can be investigated by comparing the concentration of drug required for half-maximal receptor occupancy in the presence of GTP ($D_{50\text{-GTP}}$) with the K_i value for inhibition of adenylate cyclase. The incubation media for the cyclase assay and the binding assay were the same with respect to the major constituents which influence binding properties. For those compounds which did not produce a maximum response, there was general agreement between $D_{50\text{-GTP}}$ and K_i so that it is reasonable to conclude that the level of enzyme inhibition is proportional to receptor occupancy, and efficacy is proportional to the maximum response. However, some agonists (oxo-

TABLE 4

The potency of agonists for forming the ternary complex (DRN) and drug-receptor complex (DR)

Compound	pK_{DRN}[a]	pK_{DR}[b]	$\frac{DRN_{max}}{R_T}$[c]
Oxotremorine-M	8.2	5.9	0.80
Carbachol	6.2	4.3	0.79
cis-Dioxolane	6.8	4.9	0.79
Oxotremorine	8.0	6.3	0.78
(+)-Aceclidine	6.4	4.9	0.77
(−)-Aceclidine	6.1	4.8	0.75
N-Methylaceclidine	5.6	4.7	0.70
BM5	7.5	6.6	0.68
BOK1	7.5	6.8	0.62

[a] Negative logarithm of the concentration of agonist required for half-maximal formation of the ternary complex (*DRN*).

[b] Negative logarithm of the concentration of agonist required for half-maximal formation of the drug-receptor complex (*DR*).

[c] The ratio of the maximum amount of ternary complex (DRN_{max}) formed at a saturating concentration of the agonist divided by the total amount of receptor (R_T).

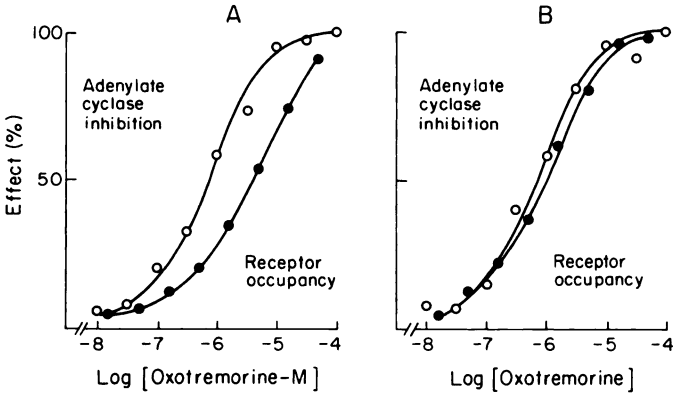

FIG. 9. *A comparison of receptor occupancy (●) and adenylate cyclase inhibition (○) for oxotremorine-M (A) and oxotremorine (B)*

Occupancy was calculated as the percentage of specifically bound [^{3}H]-(−)-NMS displaced in the presence of GTP. The occupancy curves have been corrected for the competitive shift caused by [^{3}H]-(−)-NMS. Both the occupancy and cyclase inhibition curves have been normalized with respect to the maximum effect observed at high concentrations of the agonists.

tremorine-M, *cis*-dioxolane, and carbachol) produced a maximum response when the receptors were not fully occupied. Fig. 9 shows the relationship between adenylate cyclase inhibition and receptor occupancy for oxotremorine-M and oxotremorine. Although both compounds produced a similar maximum response, oxotremorine-M caused half-maximal inhibition of adenylate cyclase activity at about 25% receptor occupancy, whereas a much closer agreement between the level of enzyme inhibition and receptor occupancy existed with regard to oxotremorine. The simplest explanation for this behavior is that oxotremorine-M is more efficacious than oxotremorine and that the maximum achievable level of enzyme inhibition plateaus at a finite level of stimulus. In order to calculate the efficacy of oxotremorine-M relative to oxotremorine, it is necessary to postulate a relationship between receptor occupancy and the measured response. The simplest assumption is that the response is proportional to the stimulus (stimulus = occupancy × efficacy) up to a ceiling level, above which there is no further increase in response with an increase in receptor occupancy. The theoretical maximum response which would have been achieved had the ceiling not been present is a useful measure of efficacy and can be calculated from the expression below which defines the relationship between two different concentrations of agonist producing equal fractional response and receptor occupancy:

$$\frac{A'}{A' + K_A} = e \times E_{max}/E_{max\text{-}A} \times \frac{A}{A + K_A} \qquad (3)$$

In this expression, E_{max} denotes the maximum response of the system, $E_{max\text{-}A}$ denotes the maximum response of the agonist, K_A denotes the dissociation constant of the agonist, A denotes the concentration of agonist producing a fractional response equal to receptor occupancy of the agonist at the concentration denoted by A', and e denotes efficacy. When A' is equal to K_A, it can be shown that

$$e = E_{max\text{-}A}/E_{max} \times (A'/A + 1) \times \tfrac{1}{2} \qquad (4)$$

in which A'/A is equal to the ratio of concentrations of agonist resulting in half-maximal receptor occupancy and half-maximal response. This equation can be used to calculate the intrinsic efficacy of agonists which produce a full maximum response as well as those which do not so long as the assumptions described above are correct. For agonists which do not produce a maximum response, the theory requires that $A'/A = 1$, so that efficacy is simply equal to

$$e = E_{max\text{-}A}/E_{max} \qquad (5)$$

Inspection of the data in Tables 1 and 2 shows that the ratio of concentrations of agonist resulting in half-maximal receptor occupancy and half-maximal response ($D_{50\text{-}GTP}/K_i$) is approximately equal to 1 for most of the compounds which do not produce a full maximum response. Since there are experimental errors in determining the ratio $D_{50\text{-}GTP}/K_i$, particularly for those compounds like BM5 and BOK1 which have very flat dose response curves for inhibition of adenylate cyclase, it was thought that the best estimate of efficacy for compounds not producing a full maximum response would be calculated by Eq. 5. The validity of this calculation is independent of the relationship between receptor occupancy and response so long as the agonist does not produce a full maximum response. The efficacies of (+)-aceclidine, (−)-aceclidine, *N*-methylaceclidine, BM5, and BOK1 were calculated in this manner using Eq. 5. For those compounds producing a maximum or near-maximum response (carbachol, *cis*-dioxolane, oxotremorine, and oxotremorine-M), efficacy was calculated according to Eq. 4, thereby taking into account the existence of a receptor reserve. The results of these calculations of efficacy are listed in Table 1.

DISCUSSION

Previous studies of the binding of muscarinic agonists have shown that agonist-binding properties are hetero-

geneous and that the nature of this heterogeneity varies from tissue to tissue (12, 13). In the rat cerebral cortex, for example, early studies showed that within the constraints of a model of independent binding sites, it was necessary to postulate at least three types of binding sites (superhigh, high, and low affinity) in order to describe agonist-binding isotherms adequately (13). In more recent studies employing the selective muscarinic antagonist [^{3}H]pirenzepine, Birdsall and coworkers (21) have presented evidence that there may be independent pools of muscarinic agonist-binding sites in rat brain, not all of which appear to be influenced by GTP. The independent nature of muscarinic agonist-binding sites in rat cerebral cortex is also displayed by the results of experiments showing a selective alkylation of agonist receptor subtypes. It has been possible to alkylate either high or low affinity agonist sites selectively in rat cortex with a 2-chloroethylamine derivative of oxotremorine (BM 123) by taking advantage of kinetic differences in the rates of alkylation of these two sites (22). It has also been possible to alkylate low affinity agonist-binding sites selectively with benzilylcholine mustard by protecting the high affinity site with a reversible agonist (23). If all of the agonist receptor subtypes in rat cortex were interconvertible, it would not be possible to alkylate these sites selectively.

Although the binding properties of muscarinic receptors in brain and perhaps other tissues may be best described by a model incorporating independent binding sites, I find the ternary complex model satisfactory for describing the binding properties of muscarinic agonists in the heart. Assuming it is correct, the ternary complex model has obvious advantages over a two-independent-site model in predicting the relative amount of bound drug in the form of ternary complex (*DRN*) and drug-receptor complex (*DR*) as shown by the results in Fig. 8. Although the two-independent-site model can be used to make tentative conclusions about different agonist states of the receptor, the ternary complex model provides a more internally consistent set of quantitative conclusions. For example, the results of numerous biochemical and binding studies on a variety of receptors are consistent with the postulate that the high affinity agonist state represents an agonist-receptor-guanine nucleotide-binding protein complex (*DRN*) and that GTP destabilizes this complex yielding a low affinity agonist-receptor complex (*DR*). Studies of agonist binding in the presence and absence of GTP are consistent with this hypothesis since GTP has been shown to convert a heterogeneous agonist competition curve into a more homogeneous one of lower overall affinity (12, 21). Often, though, the affinity of the agonist in the presence of guanine nucleotides is clearly lower than that of the low affinity component of the agonist-binding isotherm measured in the absence of GTP (12, 24), so that there does not appear to be a simple conversion of high to low affinity sites. However, in the present study, there was good agreement between the concentration of agonist required for half-maximal receptor occupancy in the presence of GTP and the reciprocal of the affinity constant ($1/K_1$) of the agonist receptor complex (*DR*) estimated by regression analysis of the agonist competition data obtained in the absence of GTP. The close agreement between the two independent estimates is shown in Fig. 10. Thus, GTP appears to reduce the positive cooperativity (α) between the binding of *N* and *D* to the receptor complex so that the binding of agonist in the presence of excess GTP is nearly homogeneous with an affinity constant approximately equal to K_1.

The ternary complex model predicts that the shape of the receptor occupancy curve for agonists depends on the concentration of *N* in the membrane. When there is an excess of *N* relative to *R*, the agonist binding curve should resemble a simple one-site model of high affinity (see Eq. A15). It is only when the availability of *N* is limiting ($N/R \simeq 1$) that the agonist occupancy curve resembles a two-independent-site model with a Hill coefficient less than 1. In the present study, the ratio N_T/R_T (β) was estimated to be 0.81. This relative estimate of the density of inhibitory guanine nucleotide-binding protein is quite small when compared with the total density of guanosine 5′-(3-*O*-thiol)-triphosphate-binding sites in bovine heart which exceeds the density of muscarinic receptors by 60- to 240-fold (see Ref. 25). An explanation for this apparent discrepancy is that perhaps only a small pool of inhibitory guanine nucleotide-binding sites interacts with muscarinic receptors in homogenates of the myocardium.

Previous studies have shown that the binding properties of [^{3}H]-(−)-NMS in the rat myocardium are heterogeneous when measured in buffers of low ionic strength containing Tris (26). Under these conditions, guanine nucleotides stimulate [^{3}H]-(−)-NMS binding by converting the heterogeneous receptor population into a homogeneous one of high affinity (26). In the absence of Tris or in Tris buffer containing 0.1 M NaCl, the binding of [^{3}H]-(−)-NMS to the myocardium is consistent with a one-site model of high affinity, and there is little or no stimulation of binding by guanine nucleotides (26). The present studies were carried out in a buffer system lacking Tris and containing 0.1 M NaCl, so that the binding

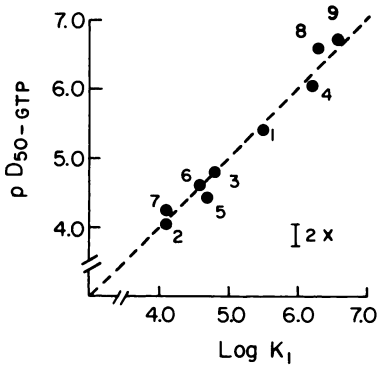

FIG. 10. *Correlation between the concentration of each drug required for half-maximal receptor occupancy in the presence of GTP and the microscopic affinity constant (K_1) of the drug receptor complex (DR) estimated in the absence of GTP*

The negative logarithm of the concentration of agonist required for half-maximal receptor occupancy in the presence of GTP ($pD_{50\text{-}GTP}$) is plotted against log K_1. The data are from Tables 2 and 3. *1*, oxotremorine-M; *2*, carbachol; *3*, *cis*-dioxolane; *4*, oxotremorine; *5*, (+)-aceclidine; *6*, (−)-aceclidine; *7*, *N*-methylaceclidine; *8*, BM5; *9*, BOK1.

properties of [^{3}H]-(−)-NMS would be homogeneous and the data analysis simple. Under these conditions, no significant effect of GTP on [^{3}H]-(−)-NMS binding was detected. It should be noted, however, that Eq. A11 can be used to analyze agonist/^{3}H-antagonist competition data even when both the agonist and antagonist discriminate between the *DR* and *DRN* complexes.

Apparently, only a very small proportion of the muscarinic receptors in the heart are coupled with *N* in the absence of an agonist. This conclusion is based on the finding that the best fit of the ternary complex model to the various agonist/[^{3}H]-(−)-NMS competition curves was achieved when K_2 was assigned a value equal to or less than 10^{-2}. It can be shown that when the value of K_2 is small, then $K_2 \simeq RN/N$. Thus, less than 1% of the muscarinic receptors in the heart appear to be precoupled with *N*. Whether this condition holds true for assay conditions other than those used in the present study is unclear. The heterogeneous binding properties of [^{3}H]-(−)-NMS noted in Tris buffer lacking NaCl (26) may be due to a substantial proportion of the receptor precoupled with *N*. If this condition is true, then the stimulation of [^{3}H]-(−)-NMS and [^{3}H]-(−)-QNB binding noted previously can be explained if these antagonists have higher affinity for R than for RN ($\gamma << 1$) as suggested by Burgisser *et al.* (27). It should be noted that, even if NMS has higher affinity for R than for RN, the NMS-binding isotherm will still obey a one-site model as long as $K_2 << 1$, and, consequently, it is still appropriate to use Eq. A14 to describe agonist/[^{3}H]-(−)-NMS competition data under these conditions. In a study of the application of the ternary complex model to the binding of agonists to the pituitary D_2-dopamine receptor, a good fit of the model to the data could be obtained only when it was assumed that a substantial proportion of the receptor was precoupled with N (15). Thus, the amount of receptor precoupled with N appears to depend on the ionic composition of the incubation medium and perhaps on the receptor type as well.

Although it was not possible to estimate the value of K_2 from the experimental data in the present report, it may be possible to do so by considering other independent findings. Berrie et al. (28) have solubilized a guanine nucleotide-sensitive agonist-binding site from rat myocardium which most likely represents the muscarinic receptor coupled to a guanine nucleotide-binding protein. They have estimated the affinity constant of oxotremorine-M for the soluble receptor to be about 10^9 M^{-1}. If this value represents a reasonable estimate of the microscopic affinity constant (αK_1) of oxotremorine-M for RN, then values of 10^{-2} and 10^4 can be calculated for K_2 and α, respectively, using approximate values of 10^2 and 10^5 for αK_2 and K_1, as shown in Table 3.

Perhaps the most convincing evidence for the application of the ternary complex model to muscarinic receptor-binding properties in the heart is the excellent quantitative agreement between the parameters of the model and pharmacological parameters. The ternary complex model predicts that the efficacy of an agonist is proportional to its ability to stabilize the ternary complex (DRN). In other words, efficacy should be proportional to the amount of positive cooperativity (α) between the binding of the agonist and N with the receptor. Although it was not possible to measure α directly in the present study, it was possible to measure αK_2. Since K_2 is a property of N and R, it is independent of the agonist. Therefore, efficacy should also be proportional to αK_2. The results in Fig. 11A show that there is excellent correlation between the value of αK_2 and the efficacy of the various muscarinic agonists as measured by inhibition of adenylate cyclase. As expected, there was also good correlation between efficacy and the negatively cooperative effects of GTP on agonist properties binding as shown in Fig. 11B. Such a relationship was observed in a previous study of the influence of guanyl-5′-yl imidodiphosphate on the binding of a series of agonists, partial agonists, and antagonists to muscarinic receptors in the longitudinal muscle of the rat ileum (29). It is apparent from the data in Fig. 8 and Table 1 that when the agonist competition curves are analyzed by a model of two independent sites, then efficacy is related to both the ratio of affinity constants of the high and low affinity sites (K_H/K_L) as well as the ratio of their relative abundances (R_H/R_L).

The various muscarinic agents examined in this study had effects on adenylate cyclase which were consistent with their reported pharmacological activity in other preparations. In the isolated guinea pig ileum (30) and in other tests of muscarinic activity (31), BOK1 behaves as a competitive antagonist, producing no effects on its own but antagonizing the action of other muscarinic agonists. In order to compare the contractile activity of muscarinic agonists on the guinea pig ileum with other muscarinic responses, it is necessary to use a pharmacological procedure, like that of Furchgott (32), to take into account the large density of spare receptors in the guinea pig ileum. Ringdahl (33, 34) has used Furchgott's method to analyze dose response data from the isolated guinea pig ileum and has estimated that the efficacy of the active enantiomer of BM5 is about one-tenth that of oxotremorine, whereas the relative efficacies of oxotremorine-M and carbachol are approximately 7-fold greater than that of oxotremorine. Fisher *et al.* (35) have examined the effects of cholinergic analogues on phosphatidylinositol turnover in guinea pig brain and have found that, at a saturating concentration (1.0 mM), BOK1 causes approximately one-tenth of the amount of incorporation of inorganic phosphate into phosphatidylinositol and phosphatidic acid as compared to oxotremorine, whereas oxotremorine-M and carbachol are about 6 times more efficacious than oxotremorine in this respect. With regard to the phosphatidylinositol effect, there appear to be little or no spare receptors, so that the efficacy of muscarinic agonists is proportional to the maximum response. Thus, the efficacy values of BOK1, BM5, oxotremorine, oxotremorine-M, and carbachol calculated in the present study (see Table 1) are in reasonable agreement with those calculated by other investigators in experiments on contractility of the isolated guinea pig ileum and on phosphatidylinositol turnover in the guinea pig cerebral cortex. The agreement among the various sets of data suggests that oxotremorine-M,

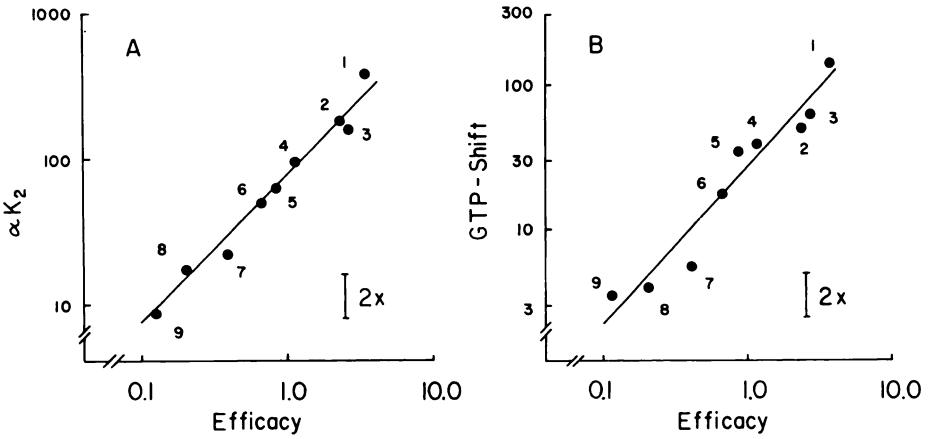

FIG. 11. *Correlation between efficacy and binding properties*

A: αK_2 is plotted against the efficacy of each agonist for inhibition of adenylate cyclase. B: The ratio of concentrations of agonist required for half-maximal receptor occupancy in the presence and absence of GTP is plotted aginst efficacy for inhibition of adenylate cyclse. The data are from Tables 1–3. Each *point* is labeled as described in the legend to Fig. 10.

oxotremorine, carbachol, and BOK1 have very little or no ability to discriminate among muscarinic receptors which inhibit adenylate cyclase in the myocardium, stimulate phosphatidylinositol turnover in the cerebral cortex, and stimulate contractions of the guinea pig ileum.

As shown here, previous studies have demonstrated that carbachol and oxotremorine have the same maximal effect on cyclic AMP metabolism (20, 36) and that carbachol causes a maximal effect at lower levels of receptor occupancy as compared to oxotremorine (20). The results of both pharmacological and binding studies have shown that aceclidine is much more active than *N*-methylaceclidine (37, 38). Similar results were observed in the present study which demonstrated that the affinity and efficacy of *N*-methylaceclidine are less than those of the enantiomers of aceclidine. Curiously, though, in the present study, the affinity and efficacy of the (+)-enantiomer of aceclidine were only slightly greater than those of the (−)-enantiomer, whereas in a variety of *in vitro* and *in vivo* tests for muscarinic activity, these stereoisomers typically display a 10- to 60-fold difference in pharmacological activity (38). Collectively, these results suggest that muscarinic receptors in the heart differ from those in other tissues insofar as they are unable to discriminate between the enantiomers of aceclidine. In the present study, *cis*-dioxolane was a potent and highly efficacious inhibitor of adenylate cyclase activity, and these results are consistent with its activity in the isolated guinea pig ileum (39).

The results described herein show that the rabbit myocardium is a very suitable model system for studying the binding properties of muscarinic agents and their effects on adenylate cyclase. Moreover, the method used in this study for analyzing the binding data according to the ternary complex model should have application to other receptors which are coupled to GTP-regulatory proteins.

ACKNOWLEDGMENT

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APPENDIX

Equations, below, are derived which describe the competitive interaction between a ^{3}H-ligand (L), a nonlabeled drug (D), and a receptor (R) that can associate with a guanine nucleotide-binding protein (N). The following scheme describes the various equilibria:

$$\begin{array}{ccccc} D & & D & & L \\ + & K_3 & + & K_1 & + \\ LR+N & \rightleftharpoons & L+R+N & \rightleftharpoons & DR+N \\ \gamma K_2 \downuparrows & & K_2 \downuparrows & & \alpha K_2 \downuparrows \\ LRN & \rightleftharpoons & L+RN & \rightleftharpoons & DRN \\ + & \gamma K_3 & + & \alpha K_1 & + \\ D & & D & & L \end{array}$$

The affinity constants of D and L for R and RN are given by:

$$K_1 = [DR]/[D][R] \quad \text{(A1)}$$

$$\alpha K_1 = [DRN]/[D][RN] \quad \text{(A2)}$$

$$K_3 = [LR]/[L][R] \quad \text{(A3)}$$

$$\gamma K_3 = [LRN]/[L][RN] \quad \text{(A4)}$$

Since the equilibrium between R and N occurs within the membrane, their local concentrations are independent of the total concentration of receptors in solution. Consequently, it is useful to normalize their respective affinity constants by multiplying them by the total receptor concentration ($[R_T]$):

$$K_2 = [R_T]\,[RN]/[N][R] \quad \text{(A5)}$$

$$\alpha K_2 = [R_T]\,[DRN]/[N][DR] \quad \text{(A6)}$$

$$\gamma K_2 = [R_T]\,[LRN]/[LR][N] \quad \text{(A7)}$$

The following conservation of mass equations apply:

$$[R_T] = [R] + [DR] + [LR] + [RN] + [DRN] + [LRN] \quad \text{(A8)}$$

$$[N_T] = [N_B] + [N_F] \quad \text{(A9)}$$

where N_T, N_B, and N_F represent total, bound, and free guanine nucleotide-binding protein, respectively. The following substitution is useful:

$$\beta = [N_T]/[R_T] \quad \text{(A10)}$$

The analytical solution to this system of equations is:

$$[LR] + [LRN] = f(L) = \frac{[L][R_T]}{([L] + K_L)} \quad \text{(A11)}$$

where

$$K_L = \frac{1 + K_1[D] + K_2[N_F](1 + \alpha K_1[D])}{K_3 + \gamma K_2 K_3[N_F]}$$

$$[N_F] = \frac{B + (B^2 + 4C)^{1/2}}{2}$$

$$B = \beta - 1 - K_N$$

$$C = \beta K_N$$

$$K_N = \frac{1 + K_3[L] + K_1[D]}{K_2 + \gamma K_2 K_3[L] + \alpha K_1 K_2[D]}$$

The function $f(L)$ is a general equation describing the binding of L in the presence of a competitive inhibitor (D) to a receptor system which conforms to the ternary complex model. The equation can be simplified to account for situations in which there is no competitive inhibitor ($D = 0$). It can also be modified to account for conditions in which there is no cooperativity between the binding of D and N ($\alpha = 1$) or between the binding of L and N ($\gamma = 1$). In the present study, evidence is presented which shows that there is no cooperativity between the binding of [^{3}H]-(−)-NMS and N. Assuming that $\gamma = 1$ and $D = 0$, Eq. A11 can be modified to describe the binding of [^{3}H]-(−)-NMS in the absence of D:

$$[LR] + [LRN] = g(L) = \frac{[L][R_T]}{([L] + 1/K_3)} \qquad \text{(A12)}$$

For analysis of drug/^{3}H-ligand competition experiments, it is useful to perform nonlinear regression analysis on data in the form of measurements of the proportion of specifically bound ^{3}H-ligand at various concentrations of the nonlabeled inhibitor. The equation describing data in this form is:

$$Y = f(L)/g(L) \qquad \text{(A13)}$$

where Y is equal to the amount of L bound specifically in the presence of D divided by that measured in the absence of D. By making the appropriate substitutions it can be shown that:

$$Y = \frac{1}{(1 + [D]/K_D(1 + [L]K_3))} \qquad \text{(A14)}$$

$$K_D = \frac{1 + K_2[N_F]}{K_1 + \alpha K_1 K_2[N_F]}$$

In this equation, $[N_F]$ is defined as it is in Eq. A11.

It is informative to calculate the theoretical proportions of bound drug in the form of DR and DRN. Using strategy similar to that shown above for the binding of L, it can be shown that the equation describing the binding of D is:

$$\frac{[DR] + [DRN]}{[R_T]} = \frac{D}{D + K_d} \qquad \text{(A15)}$$

where

$$K_d = \frac{1 + K_2[N_F]}{K_1 + \alpha K_1 K_2[N_F]}$$

$$[N_F] = \frac{b + (b^2 + 4c)^{1/2}}{2}$$

$$b = \beta - 1 - K_N'$$

$$c = \beta K_N'$$

$$K_N' = \frac{1 + K_1[D]}{K_2 + \alpha K_1 K_2[D]}$$

The equation describing the amount of ternary complex formed is:

$$\frac{DRN}{[R_T]} = \frac{D}{D + K_{drn}} \qquad \text{(A16)}$$

$$K_{drn} = \frac{1 + K_1[D] + K_2[N_F]}{\alpha K_1 K_2[N_F]}$$

In this equation, $[N_F]$ is defined as it is in Eq. A15. The amount of bound drug in the form of DR can be calculated by taking the difference between Eqs. A15 and A16. The theoretical curves describing bound drug in the form of DR and DRN were calculated using the parameter estimates in Table 3 and Eqs. A15 and A16. The concentration of drug causing half-maximal formation of DRN and DR and the theoretical maximum value of DRN/R_T were estimated graphically.

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