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# Reciprocal modulation of agonist and antagonist binding to muscarinic cholinergic receptor by guanine nucleotide

(frog myocardium/radioligand binding)

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**ABSTRACT** **The ability of guanine nucleotide to decrease the binding affinity of agonists but not antagonists has been documented in a number of hormone and neurotransmitter receptor systems. By contrast, recent reports indicate that both agonist *and* antagonist binding to the muscarinic cholinergic receptors appear to be regulated in a reciprocal fashion by guanine nucleotide. We document two forms of the muscarinic cholinergic receptor in frog heart, which are present in approximately equal proportions and which display high-agonist/low antagonist and low-agonist/high-antagonist affinities, respectively. Guanine nucleotide appears to convert the former type of site into the latter type. These observations can be interpreted in terms of a model for two interconvertible forms of the muscarinic cholinergic receptor reciprocally favored by agonists and antagonists. This model has implications both for the understanding of neurotransmitter–receptor interactions generally and for the nature of the biological effects of receptor antagonists.**

Agonist binding to receptors shows properties distinct from those observed with antagonists. For example, a selective modulatory effect of guanine nucleotide on the high-affinity form of agonist binding but not on antagonist binding to receptors, extensively documented in β-adrenergic receptor systems (1, 2), also has been found in many other neurotransmitter receptor systems (3–7).

The widespread occurrence of these agonist-specific binding properties has led to the proposal that they reflect early agonist-induced activation steps leading to the cellular response to the drug stimulus. Parallel studies of β-adrenergic receptor systems by several techniques (8–10) support the notion that agonist and guanine nucleotide sequentially promote the formation and the processing of a complex between the receptor and a guanine nucleotide regulatory protein (9), providing an explanation for the observed agonist-specific binding properties. Antagonists, which occupy receptor binding sites without eliciting a response, are devoid of these binding modulatory effects of guanine nucleotides at β-adrenergic receptors.

In contrast, recent observations have indicated that, in addition to a guanine nucleotide-induced transition of the agonist–receptor complex from a high-affinity form to a low-affinity form, muscarinic cholinergic receptors in rat heart (4), brain (11, 12), and ileum (11, 12) show an enhancing effect of guanine nucleotides on antagonist binding. Studies of antagonist binding in cardiac (13) and striatal (14) membranes under conditions of low ionic strength have suggested the presence of two forms of the muscarinic cholinergic receptor with high and low affinity for antagonists. Whether or not these two forms of the receptor, discriminated by antagonists, could relate to the two forms of the same receptor discriminated by agonists was not ascertained.

In this communication, we describe the results of the quantitative analysis of the effect of guanine nucleotide on agonist and antagonist binding according to models for one or several forms of the muscarinic cholinergic receptor sites. The interpretation of these results is discussed in terms of potential models for drug–receptor interactions.

## METHODS

The radioligand (−)-[$^3$H]quinuclidinyl benzylate ([$^3$H]QNB) (40.2 Ci/mmol; 1 Ci = $3.7 \times 10^{10}$ becquerels) was obtained from New England Nuclear. (−)-QNB was a gift from Hoffman La Roche, oxotremorine was purchased from Sigma, and 5′-guanylyl imidodiphosphate (p[NH]ppG) was either from Sigma or Boehringer. The sources and purity of other materials used in this study have been reported (15).

Whole frog hearts (*Rana pipiens*) were frozen in liquid nitrogen after removal and stored at −80°C. Preparation of frog heart membranes was as follows. Frozen hearts (100 g) were thawed in buffer A (5 mM Tris·HCl/1 mM $MgCl_2$/0.25 M sucrose, pH 7.4), fragmented with scissors, and homogenized three times for 5 sec with a Brinkman Polytron at a low-speed setting. The homogenate was filtered through cheesecloth and homogenized with five strokes of a tightly fitting Potter–Elvehjem homogenizer. The homogenate was then centrifuged for 10 min at 3,000 × g, and this was repeated once with the resultant supernatant. The second supernatant obtained at low speed was then centrifuged for 10 min at 39,000 × g. The pellet obtained at high speed was resuspended in buffer B (75 mM Tris·HCl/25 mM $MgCl_2$, pH 7.4). This procedure was repeated twice, and finally the pellet was resuspended in 200 ml of buffer B (5 μM phenylmethylsulfonyl fluoride/1 mM dithiothreitol/0.25 M sucrose). This was frozen in liquid nitrogen and stored at −80°C. No difference in agonist and antagonist binding was observed between fresh or frozen membranes. All steps described above were carried out at 0–6°C. Typically, the membranes contained 320 fmol of (−)-[$^3$H]QNB binding sites per mg.

Equilibrium binding studies were performed by a filtration method with Whatman GF/C filters as described (16). The incubation was at 25°C for 60 min in 50 mM Tris, pH 7.4/10 mM $MgCl_2$/1 mM EDTA (final assay volume, 1 ml). Doubling the incubation period to 120 min did not significantly alter the re-

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Abbreviations: QNB, quinuclidinyl benzylate; p[NH]ppG, 5′-guanylyl imidodiphosphate.

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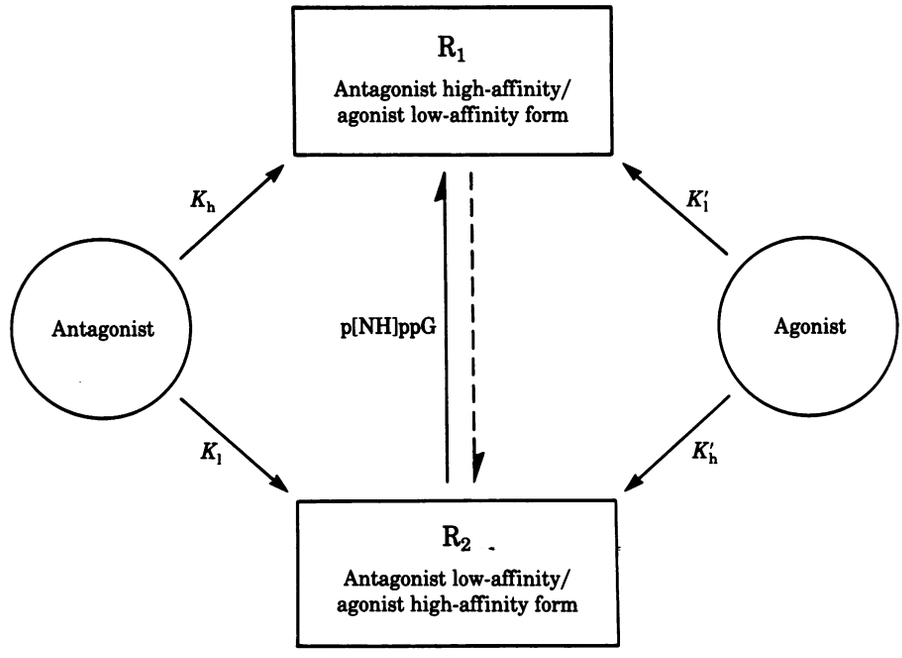


FIG. 1. Schematic diagram of the reciprocal binding model showing two distinct forms $R_1$ and $R_2$ of the receptor distinguished by both antagonists and agonists. The form $R_1$ has a higher affinity ($K_h$) for antagonists than does the form $R_2$; reciprocally, $R_2$ has more affinity ($K'_h$) for agonists than does $R_1$. The two forms are interconvertible in the presence of guanine nucleotide (e.g., p[NH]ppG). $K_l$, low affinity.

sults, indicating that the binding was at steady state. Nonspecific binding was defined as the binding of (−)-[$^3$H]QNB in the presence of 1 $\mu$M (−)-QNB and was <10% of the total binding in all cases.

Data was analyzed with the aid of a weighted nonlinear least-squares computer curve-fitting method according to the Law of Mass Action (9, 17). Various models were successively used, and the goodness of the fit was statistically compared among them to determine the most appropriate model ("best fit"). The models used are based on the assumption that deviations of the binding curve from that expected for a bimolecular reaction are due to differential affinities of the ligand for separate forms of the receptor sites (Fig. 1). In the case of (−)-[$^3$H]QNB saturation binding experiments, the model involves the binding of the radioligand to two distinct forms of the receptor ($R_1$, $R_2$) for which the tracer has different affinity (i.e., the dissociation constant $K_{d_1} < K_{d_2}$). For competition binding curves of the agonist oxotremorine versus (−)-[$^3$H]QNB, the model ("reciprocal" model) involves competitive binding of the radioligand and the competitor to the same forms of the receptor ($R_1$, $R_2$) for which agonist differential affinity (i.e., the dissociation constant $K'_{d_1} > K'_{d_2}$) is reciprocal to antagonist affinity ($K_{d_1} < K_{d_2}$). Both forms ($R_1$ and $R_2$) of the receptor are distinct, but their relative proportion can vary under conditions favoring a transition of one form into the other. The reciprocal model (Fig. 1) is an extension of a model for two forms of the receptor exclusively distinguished by agonists previously documented in several receptor systems (3, 9, 13, 15). It incorporates the additional property of different affinities of antagonists for both forms of the receptor in an order reciprocal to those for agonists. Estimates of the slope of competition curves also were analyzed by a four-parameter logistic equation as described (18). A slope of 1 is compatible with competitive interactions of the ligands with a single form of receptor sites.

## RESULTS

In the absence of guanine nucleotide, detailed saturation binding experiments with the muscarinic antagonist ligand (−)-[$^3$H]QNB in frog heart membranes demonstrated deviations from a simple bimolecular binding isotherm. When such curves were analyzed according to a model for two separate forms of the receptor (two-site model), a significant improvement ($P < 0.05$) of the goodness of the fit was obtained (Fig. 2). The binding parameters from the experiment shown in Fig. 2 are summarized in Table 1. The extent of the effect of guanine nucleotides on antagonist binding was somewhat variable. In all experiments, however, the binding isotherm for (−)-[$^3$H]QNB was significantly better explained by a model for two forms of the receptor in the absence of guanine nucleotides. In a series of six such experiments, (−)-[$^3$H]QNB had a lower affinity (by a factor of ≈17) for one form of the receptor, $R_2$ ($K_{d_2} = 773 \pm 252 \times 10^{-12}$M, $n = 6$), than for the other form of the receptor sites, $R_1$ ($K_{d_1} = 46 \pm 5 \times 10^{-12}$M, $n = 6$).¶ On the average, both forms $R_1$ and $R_2$ were found in equal percentages (%$R_1 = 47 \pm 6$, $n = 6$). Addition of guanine nucleotides (1 mM GTP or 0.1 mM p[NH]ppG) to the incubation mixture led to a significant increase in (−)-[$^3$H]QNB binding (Fig. 2). Saturation curves were now best fit by a model for a single form R of the receptor (one-site model) with an affinity for the radioligand ($K_d = 44 \pm 5 \times 10^{-12}$, $n = 6$) which was not statistically different from that of the higher-affinity form observed in the absence of nucleotide. Moreover, the sum of the concentration of high- and low-affinity binding sites observed in the absence of nucleotide was not statistically different from the concentration of high-affinity sites observed in the presence of guanine nucleotide (Table 1).

We also examined the effect of p[NH]ppG on the binding of the antagonist (−)-QNB to its receptors by studying (−)-[$^3$H]QNB competition curves versus (−)-QNB. As expected from the results shown in Fig. 2, the upper plateau of the curve was much higher when guanine nucleotide was present (Fig.

¶ A geometric mean is reported for averaged dissociation constants because estimates for $K_d$s tend to be log normally distributed, as we have recently demonstrated. In contrast, averages of estimates for receptor site proportions are arithmetic means because estimates for Rs appear to be normally distributed (unpublished data).

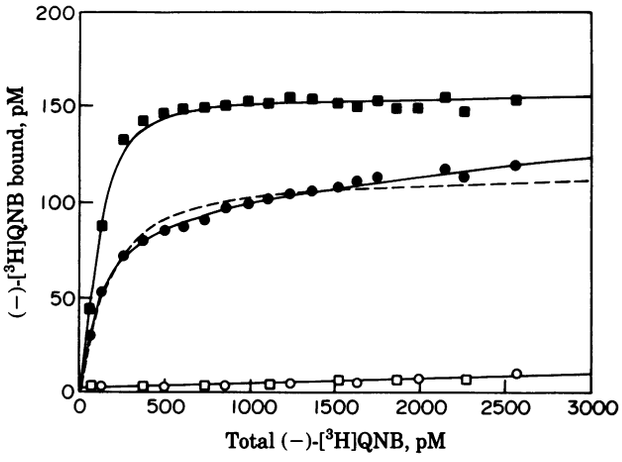


FIG. 2. (−)-[$^3$H]QNB saturation curve in frog heart membranes in the absence (●) and presence (■) of p[NH]ppG. In the absence of p[NH]ppG, a significant improvement in the computer fit ($P < 0.001$) was obtained when a two-site model was assumed (●——●) compared with the best computer fit for a one-site model (●– – –●). There was no improvement for the two-site model when 0.1 mM p[NH]ppG was present. Specific and nonspecific binding are shown. The values for the binding parameters are given in Table 1.

3). The lines through the data points were computer fitted by assuming a two-site model in the absence and a one-site model in the presence of p[NH]ppG, with no change in the total binding capacity. Normalization of competition binding curves (Fig. 3 *Inset*) obscured the effect of guanine nucleotide on antagonist binding. In the normalized format, the slope of the control curve was 0.9 ± 0.08 and steepened to a slope of 1.0 ± 0.07 in the presence of p[NH]ppG. A slight (2-fold) left shift in the curve was observed. A quite similar apparent increase of antagonist potency with guanine nucleotide also was observed by Berrie *et al.* (4) in the case of another muscarinic cholinergic antagonist ligand, [*N-methyl*-$^3$H] scopolamine methyl chloride.

We next explored the influence of guanine nucleotide on agonist competition with (−)-[$^3$H]QNB. Because the radioligand labels distinct forms of the receptor with different affinity, a proper analysis of the competition curves requires explicit inclusion of distinct affinity constants ($K_{d_1}$ and $K_{d_2}$) for (−)-[$^3$H]QNB into the model. Fig. 4 shows competition curves of the cholinergic agonist oxotremorine in the absence and presence of graded concentrations of p[NH]ppG. In the absence of guanine nucleotide, the agonist competition curve was shallow with a slope of 0.48, compatible with more than one form of the receptor with different affinity for agonists. Increasing concentrations of the guanine nucleotide caused a progressive shift to the right and steepening of the agonist competition curve. However, even in the presence of a high concentration of p[NH]ppG (up to 0.1 mM), the slope did not reach unity (0.79), and the competition curve remained biphasic, suggesting that there was still some heterogeneity of the sites present. Among several models tested, the best fit to the experimental data was obtained with a reciprocal model for agonist and antagonist binding (shown in Fig. 1). The reciprocal model postulates two forms of the muscarinic cholinergic receptor—one with high affinity for antagonist and low affinity for agonist and the other with low affinity for antagonist and high affinity for agonist (Fig. 1). Because nucleotide caused no change in total receptor number, the data suggests that guanine nucleotide initiates a conversion of low-affinity antagonist/high-affinity agonist sites into high-affinity antagonist/low-affinity agonist sites. The binding parameters for oxotremorine derived from computer analysis of Fig. 4 by the reciprocal model are summarized in Table 2. In three separate experiments in which several competition curves for oxotremorine were obtained in the presence of various concentrations of guanine nucleotide, the agonist consistently displayed about a 28-fold higher affinity for $R_2$ ($K'_{d_2} = 0.031 \pm 0.006 \times 10^{-6}$ M, $n = 10$ curves) than for $R_1$ ($K'_{d_1} = 0.86 \pm 0.09 \times 10^{-6}$ M, $n = 10$ curves). On the average, p[NH]ppG elicited an increase in $R_1$ from 35 ± 6% to 80 ± 5% of the total receptor population in these experiments.

## DISCUSSION

The major finding of the present work is that the enhancement by guanine nucleotides of antagonist binding at muscarinic cholinergic receptors reciprocally reflects the guanine nucleotide-induced transition between the two forms of the receptor discriminated by agonists.

The ability of muscarinic agonists to distinguish two forms of the receptor and of guanine nucleotide to modulate their affinity has been documented (4, 19). The effects previously demonstrated in rat heart membrane preparations were especially pronounced (4). The observation of changing proportions of the high- and low-affinity forms of the receptor supports the notion of the interconvertibility of these forms in agreement with similar findings in β-adrenergic receptor systems (17). However, in contrast to the β-adrenergic systems, a high concentration of guanine nucleotide did not promote a complete transition to a single low-affinity form of the muscarinic receptor; thus, about 20% of the sites remained in a form that bound the agonist with high affinity. This might represent a subpopulation of sites that are resistant to nucleotide effects. Alternatively, the high concentration of p[NH]ppG used might still lead to an incomplete transition of the sites into the low-affinity form for agonists.

The previous use of the racemic radioligand (±)-[$^3$H]QNB would have compromised the ability to demonstrate two forms of the receptor. We have shown that the use of such high-affinity racemic radioligands is associated with deviations from a bi-

Table 1. Parameter estimates for the models used for (−)-[$^3$H]QNB binding to frog heart membranes in the absence and presence of p[NH]ppG

| Model | p[NH]ppG | $K_{d_1}$, M × $10^{12}$ | $K_{d_2}$, M × $10^{12}$ | $R_1$ pM | $R_1$ % | $R_2$ pM | $R_2$ % |
|---|---|---|---|---|---|---|---|
| One-site | − | 102 ± 11 | — | 113 ± 2 | 100 | — | 0 |
| Two-site* | − | 52 ± 6 | 2,600 ± 1,400 | 85 ± 5 | 54 | 73 ± 15 | 46 |
| One-site | + | 30 ± 4 | — | 155 ± 1 | 100 | — | 0 |

The binding parameters obtained from the experiment in Fig. 2 are given ±SE. $K_{d_1}$ and $K_{d_2}$ are the dissociation constants, and $R_1$ and $R_2$ are the two forms of the receptor binding QNB with high and low affinity, respectively (Fig. 1).

* A two-site model for the curve in the absence of p[NH]ppG yielded a highly significant ($P < 0.001$) improvement in the computer fit when compared with a one-site model. When present, p[NH]ppG was at 0.1 mM. The experiment shown was replicated six times with comparable results.

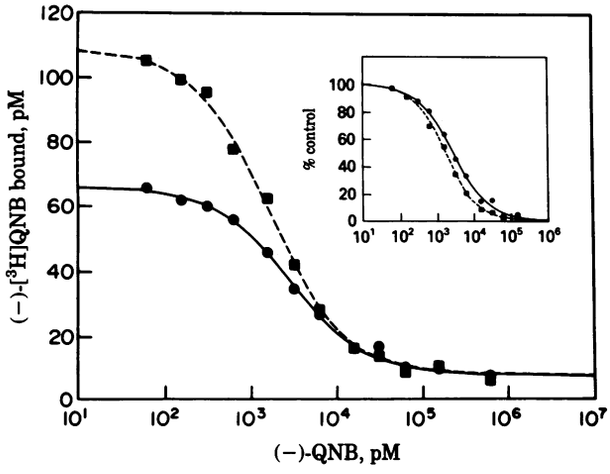


FIG. 3. (−)-QNB competition versus (−)-[³H]QNB binding in frog heart membranes in the absence (●) and presence (■) of p[NH]ppG. The (−)-[³H]QNB concentration was 1.5 nM, and the total receptor concentration was 108 pM as determined by computer analysis. The curve in the absence of p[NH]ppG was analyzed by a two-site model assuming identical properties of (−)-QNB and (−)-[³H]QNB. The curve in the presence of p[NH]ppG was analyzed by a one-site model (high affinity). (*Inset*) The same experimental data but normalized. The slopes were 0.90 ± 0.08 and 1.01 ± 0.07 for the control curve and the p[NH]ppG curve, respectively.

molecular reaction (20) because of the binding of both stereoisomers to the receptors. Using the racemic radioligand would have masked the presence of the two distinct affinity states for QNB binding (confirmed experimentally and by computer simulations). In the experiments presented here, we have utilized only the pure (−) stereoisomer of [³H]QNB and have reproducibly documented a statistically significant improvement in the fit of saturation curves with a two-site as opposed to a one-site model in the absence of guanine nucleotide. We also have been able to document this effect for (−)-[³H]QNB binding to membranes prepared from rat heart and rat brain, although the extent of modulation of antagonist binding by guanine nucleotide was less pronounced (data not shown). Comparison of the affinity of (−)-[³H]QNB for both forms of the receptor indicates that the higher affinity ($K_{d_1}$ = 46 × $10^{-12}$ M) more closely

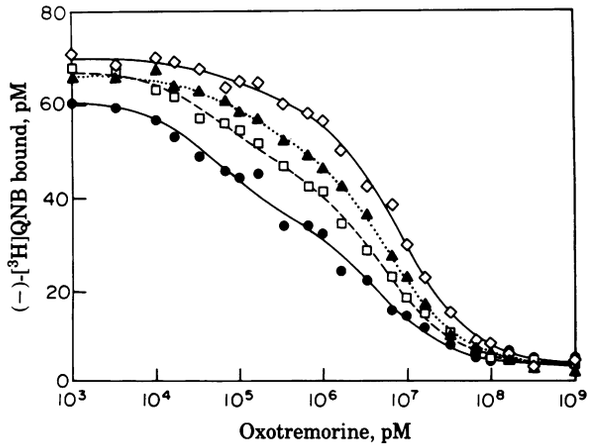


FIG. 4. Oxotremorine competition curves with (−)-[³H]QNB in the presence of graded p[NH]ppG concentrations in frog heart membranes. The curves without p[NH]ppG (●) and with p[NH]ppG at 0.01 μM (□----□), 0.1 μM (▲·····▲), and 10 μM (◇——◇) were analyzed by a reciprocal binding model (Fig. 1). The (−)-[³H]QNB concentration was 370 pM. $K_d$s and receptor concentrations are given in Table 2.

Table 2. Parameter estimates for the models used for oxotremorine binding to frog heart membrane muscarinic cholinergic receptors in the absence and presence of p[NH]ppG

| p[NH]ppG addition, μM | $K'_{d_2}$, M × $10^6$ | $K'_{d_1}$, M × $10^6$ | $R_2$ pM | $R_2$ % | $R_1$ pM | $R_1$ % |
|---|---|---|---|---|---|---|
| None | 0.028 ± 0.011 | 0.40 ± 0.08 | 86 ± 9 | 70 | 37 ± 3 | 30 |
| 0.01 | 0.043 ± 0.012 | 0.62 ± 0.06 | 79 ± 6 | 62 | 48 ± 2 | 38 |
| 0.1 | 0.088 ± 0.019 | 0.84 ± 0.04 | 57 ± 4 | 50 | 56 ± 1 | 50 |
| 10 | 0.064 ± 0.053 | 0.99 ± 0.09 | 33 ± 8 | 33 | 67 ± 2 | 67 |

The binding parameters derived from oxotremorine competition curves versus (−)-[³H]QNB binding are given ±SE. The experimental data are shown in Fig. 4. A reciprocal binding model was applied in which the dissociation constants for the radioligand ($K_{d_1}$ = 46 × $10^{-12}$ M, $K_{d_2}$ = 773 × $10^{-12}$ M) were the averaged parameter values obtained from six separate saturation experiments. The high- and low-affinity sites for (−)-[³H]QNB correspond to the low- and high-affinity sites for oxotremorine, respectively. The experiment was replicated three times with comparable results. $R_1$ is the form of the receptor that binds agonist with low affinity and antagonist with high affinity. $R_2$ is the form of the receptor with high-agonist/low-antagonist affinity. $K'_{d_1}$ and $K'_{d_2}$ are the affinities of oxotremorine (agonist) for the two forms of the receptor (Fig. 1).

matches the physiological $EC_{50}$ (10 pM) observed in guinea pig myocardium (21).

Complex radiolabeled antagonist-binding kinetics have been reported for several muscarinic receptor systems (22, 23). These binding kinetic properties have been interpreted in terms of a two-step model of a fast initial binding step followed by an antagonist-promoted receptor conformational change (22, 23). The notion of antagonist-induced receptor isomerization is compatible with our model for two interconvertible forms of the receptor. However, a two-step model fails to explain the observation of two classes of sites in antagonist saturation curves and, rather, predicts saturation curves compatible with a single class of sites (9, 24), in contrast to our experimental results (Figs. 2 and 4). Various forms of "dual receptor" models also have been popularized by Ariens (25). Those models are based on the concept that antagonists and agonists have reciprocal preferential affinity for two freely interconvertible forms (R inactive, R* active) of the receptor. Although the "reciprocal" model proposed in this communication might superficially resemble Ariens' models, clear distinctions are to be made. Ariens' models predict simple (one site) saturation curves and steep competition curves (9, 24). However, our results would be compatible with a model for two interconvertible forms of the receptor, involving interaction of the receptor with another membrane component, as recently shown for the β-adrenergic receptor (9).

Another potential explanation for the effect of guanine nucleotide on antagonist binding could be the release of endogenous agonist tightly bound to the high-affinity form of the muscarinic receptor. In this hypothesis, guanine nucleotide would elicit a selective increase in the binding capacity of the receptor without changing the apparent affinity of the radioligand for the receptor by promoting the dissociation of the putative tightly bound endogenous agonist. However, these predictions are in contrast with the data in Fig. 2 and, therefore, the hypothesis is not supported by our observations.

In conclusion, the demonstration of directionally opposite changes in muscarinic cholinergic agonist and antagonist binding with guanine nucleotide and the applicability of a reciprocal model to agonist competition curves (Fig. 4) strongly suggests that these changes are functionally linked. The present findings for the muscarinic cholinergic receptor appear to incorporate and to extend the observation in a variety of other neurotrans-

mitter receptor systems of distinct high- and low-affinity forms for agonists. Numerous receptor systems are characterized by the existence of different conformational states, with biologically active agonists stabilizing one form ($R_2$; Fig. 1) and biologically inactive antagonists displaying no preferential affinity for either form. However, in some systems such as the muscarinic cholinergic and perhaps other receptor systems, antagonists may tend to stabilize the other ($R_1$; Fig. 1) conformational state of the receptor. These conclusions are further supported by our recent observation of the same reciprocal modulatory effect of guanine nucleotides on agonist and antagonist binding to the dopaminergic receptor (unpublished data). In such systems, antagonists might be expected to exert biological actions opposite to those of agonists. Further studies will be necessary to assess this hypothesis and to determine the potential physiological significance of such nucleotide effects in muscarinic cholinergic systems and in other systems.

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