0026-895X/90/010148-11$02.00/0

MOLECULAR PHARMACOLOGY, 38:148-158

Signaling through the Muscarinic Receptor-Adenylate Cyclase System of the Heart Is Buffered against GTP over a Range of Concentrations

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Received October 9, 1989; Accepted April 24, 1990

SUMMARY

The influence of GTP on muscarinic receptor occupancy and inhibition of adenylate cyclase activity was investigated in well washed homogenates of the rat myocardium. In these homogenates, the highly efficacious muscarinic agonist oxotremorine-M was without effect on adenylate cyclase activity in the absence of exogenous GTP but caused a maximal 38% inhibition of the enzyme in the presence of 0.1 μM GTP. Increasing the concentration of GTP to 0.1 mM caused small to moderate increases in the maximal inhibition of adenylate cyclase elicited by oxotremorine-M and in the concentration of this agonist required for half-maximal inhibition of the enzyme. In contrast, the same change in the concentration of GTP (0.1 μM to 0.1 mM) caused a relatively large increase (46-fold) in the concentration of oxotremorine-M necessary for half-maximal receptor occupancy. Similar observations were made for the highly efficacious muscarinic agonist carbachol. Our results show that GTP increases receptor coupling efficiency and decreases agonist affinity and that these two effects oppose one another, so that the level of muscarinic agonist-mediated inhibition of adenylate cyclase activity remains relatively constant over a range of concentrations of GTP. We have also used a model to predict the influence of GTP on receptor binding properties and agonist-mediated inhibition of adenylate cyclase activity and have calculated theoretical results generally consistent with the experimental observations.

A variety of receptors trigger cellular responses by signaling through G proteins. Two consequences of this signaling mechanism that have been clearly established for receptors coupled to adenylate cyclase include the requirement for GTP for receptor-effector coupling and the reduction in agonist binding by GTP [see review by Dohlman *et al.* (1)]. When the dynamics of receptor-G protein coupling are considered from a pharmacological viewpoint, it can be seen that GTP is essential for the manifestation of the intrinsic efficacy of the agonist-receptor complex, because in the absence of GTP an agonist cannot trigger a response. It can be reasoned further that an increase in the concentration of GTP should increase the coupling efficiency of the agonist-receptor complex, such that the agonist would behave as if it had greater intrinsic efficacy. However, as described above, an increase in the concentration of GTP also reduces the binding affinity of agonists. Thus, it would seem likely that the effect of GTP on coupling efficiency would oppose its action on affinity, so that the level of the response generated by a given concentration of a highly efficacious agonist would remain relatively constant over a range of concentrations of GTP.

In the present study, we have put this latter postulate to a specific test by comparing the effects of GTP on the occupancy of muscarinic agonists and their inhibition of adenylate cyclase activity. The muscarinic receptor-adenylate cyclase system of the rat heart was used in our studies because it is a convenient model system that exhibits properties characteristic of a G protein-linked receptor system. For example, activation of muscarinic receptors in homogenates of the myocardium causes a pertussis toxin-sensitive (2) GTP-dependent inhibition of adenylate cyclase activity (3), and the binding of agonists to cardiac muscarinic receptors is inhibited by GTP (4, 5) in a manner that is highly dependent on the efficacy of the agonist (6). Moreover, the binding properties of the mammalian cardiac muscarinic receptor have been well characterized and exhibit characteristics of a homogeneous population of M_2 muscarinic receptors (7). Our results show that increasing the concentration of GTP from approximately 0.1 μM to 0.1 mM causes a 46–67-fold reduction in the binding affinities of the highly efficacious muscarinic agonists oxotremorine-M and carbachol, while

This work was supported by National Institutes of Health Grant NS 26511, a Research Starter Grant from the Pharmaceutical Manufacturers Association Foundation, a Grant-in-Aid from the American Heart Association, California Affiliate, and funds contributed by the American Heart Association, Chapters.

[1] Recipient of a United States Public Health Service Research Career Development Award (NS 01396) from the National Institute of Neurological Disorders and Stroke.

ABBREVIATIONS: G protein, GTP-binding protein; NMS, *N*-methylscopolamine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

having only modest effects on the ability of these agonists to inhibit adenylate cyclase activity. We have also used a simplified model based on the "ternary complex model" of De Lean *et al.* (8) to predict the influence of GTP on a G protein-linked receptor system and have obtained theoretical results generally consistent with the experimental observations. The consequences of our model are inconsistent with some intuitive ideas about how the binding of an agonist to a receptor system conforming to the ternary complex model should behave in the presence of GTP. Our results have implications for a variety of receptors that signal through G proteins and for experiments in which null methods are used to estimate the affinities of agonists from pharmacological data.

Materials and Methods

Tissue preparation. Male Sprague Dawley rats (200–250 g) were killed by decapitation, and their hearts were removed rapidly and perfused through the aorta with ice-cold saline. The tissue was minced with scissors and homogenized with a Polytron (Brinkmann Instruments, Westbury, NY) in a buffer containing 30 mM NaHEPES, pH 7.5, and 0.25 M sucrose. The homogenate concentration was approximately 5% (w/v), based on the original wet weight. The homogenate was filtered through three layers of cheesecloth and centrifuged at 30,000 × *g* for 10 min. The supernatant was discarded, and the pellet was resuspended in fresh buffer. In order to remove most of the endogenous GTP from the cardiac homogenate, the pellet was washed two more times by centrifugation and resuspension in fresh buffer, as just described. The final pellet was resuspended to a concentration of 50 mg of tissue (original wet weight)/ml of buffer containing 30 mM NaHEPES, pH 7.5, and 0.25 M sucrose and was used immediately in both the adenylate cyclase assay and the radioligand binding assay.

Adenylate cyclase assay. Washed myocardial homogenate (0.05 ml) was incubated at 25° for 6 min in a final volume of 0.2 ml containing 30 mM NaHEPES, pH 7.5, 100 mM NaCl, 63 mM sucrose, 0.5 mM EGTA, 0.5 mM dithiothreitol, 5.0 mM $MgCl_2$, 1.0 mM cyclic AMP, 0.5 mM isobutylmethylxanthine, 0.01 mM ATP, 1 to 0.5 μCi of [α-^{32}P]ATP, 5.0 mM creatinine phosphate, 30 units/ml creatinine phosphokinase, 0.5% bovine serum albumin, and various concentrations of GTP and muscarinic agonists, as described in Results. The reaction was started by addition of tissue and was stopped by addition of 0.1 ml of a solution of 40 mM ATP, 1.4 mM cyclic AMP, and 0.1 mM sodium dodecyl sulfate, titrated to pH 7.5 with Tris base. An aliquot of water (0.8 ml) was added, and [^{32}P]cAMP was recovered by sequential chromatography over Dowex AG 50W-X4 and alumina, essentially as described by Salomon *et al.* (9). [^{32}P]cAMP in the eluate from the alumina columns was measured by Cerenkov counting. The recovery of cyclic AMP from the columns was determined to be constant at 78% in separate experiments using [^{3}H]cAMP as an internal standard.

Radioligand binding assay. The binding of the specific muscarinic antagonist [^{3}H]NMS (85 Ci/mmol; DuPont-New England Nuclear, Boston, MA) was measured by the rapid filtration technique similar to that described previously (10). Myocardial homogenate (0.1 ml) was incubated for 10 min at 25°, in a final volume of 0.4 ml containing 0.5 nM [^{3}H]NMS and all of the ingredients of the adenylate cyclase assay described above. Membrane-bound [^{3}H]NMS was trapped by rapid filtration of the incubation mixture over glass fiber filters (GF/B; Whatman, Inc., Clifton, NJ), using a cell harvester (Brandel, Gaithersburg, MD). The filters were rinsed with three aliquots (3 ml each) of ice-cold saline. All assays were done in duplicate, and nonspecific binding was defined as the residual binding in the presence of 10 μM atropine.

Receptor modeling. In order to explain how GTP modified the ability of agonists to inhibit adenylate cyclase activity and to bind with muscarinic receptors, we investigated the theoretical consequences of the model shown in Fig. 1. This model was derived by expanding the

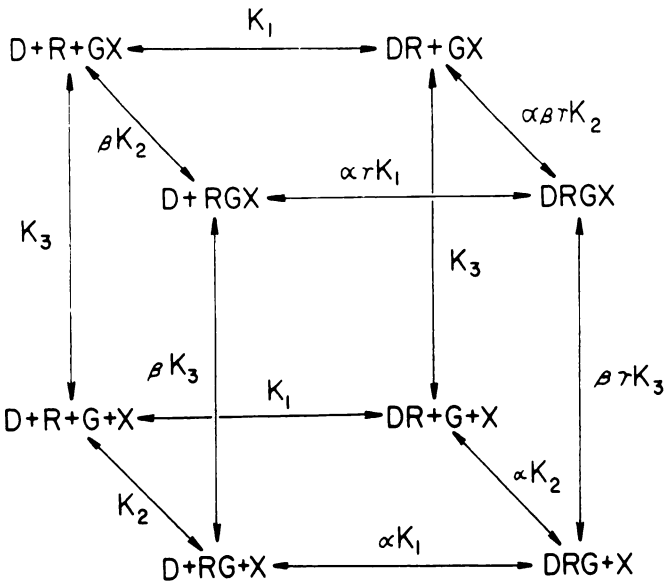

Fig. 1. Equilibria for the interaction of an agonist (*D*) with its receptor (*R*) and G protein (*G*), which can bind GTP (*X*). Further details are given in the text.

ternary complex model (8) to account for the effects of GTP on the binding of agonists. This model is based on the assumption that the agonist (*D*) binds to the recognition site on the receptor (*R*) with a dissociation constant K_1 and that GTP (*X*) binds to its recognition site on the G protein (*G*) with a dissociation constant K_3. Moreover, *R* and *G* can diffuse within the plane of the membrane and bind with each other, as described by the constant K_2. Because *R* and *G* reside in the membrane, their local concentrations are independent of the total concentration of receptors in solution. Thus, it is useful to divide the dissociation constant for their equilibrium by the total receptor concentration (i.e., $K_2 = [R][G]/[RG]R_T$). Fig. 1 shows the equilibria for all possible combinations of ligand-protein complexes (i.e., *DR*, *RG*, *GX*, *DRG*, *RGX*, and *DRGX*). Such a system abundantly displays cooperative interactions, and it can be shown that these interactions must be reciprocal; otherwise, the system would not reach equilibrium. These reciprocal interactions are a consequence of the law of conservation of the standard free energy, which has been described in detail elsewhere (11). For example, if *G* increases the affinity of *D* for *R*, then *D* must increase the affinity of *G* for *R* by precisely the same amount. The magnitude of this reciprocal change in affinity is denoted by α in Fig. 1. The other relevant cooperativity terms are denoted by β and γ. The solution to the model shown in Fig. 1 is given in the Appendix.

Calculations. The concentration of agonist causing half-maximal inhibition of adenylate cyclase activity (EC_{50}) and the maximal fractional inhibition of adenylate cyclase activity (I_{max}) caused by the agonist were estimated by nonlinear regression analysis of the data according to the following logistic equation:

$$v = P\left(1 - \frac{I_{max}X^n}{X^n + EC_{50}{}^n}\right) \quad (1)$$

where *v* denotes measurements of adenylate cyclase activity, *P* denotes the estimate of adenylate cyclase activity in the absence of agonist (*X*), and *n* denotes the Hill coefficient. The concentration of agonist yielding half-maximal receptor occupancy (X_{50}) was calculated from the concentration of agonist causing 50% displacement of specific [^{3}H]NMS binding (IC_{50}), using the following equation:

$$X_{50} = \frac{IC_{50}}{1 + [[^3H]NMS]/K_{NMS}} \quad (2)$$

in which K_{NMS} denotes the dissociation constant of [^{3}H]NMS, which was determined independently from NMS/[^{3}H]NMS competition ex-

periments. The agonist/[^{3}H]NMS competition curves measured in the presence of 0.1 mM GTP were analyzed according to a two-site model, as described previously (10).

Results

Muscarinic inhibition of adenylate cyclase. Initial experiments were run to determine the concentration range of GTP over which muscarinic receptor-mediated inhibition of adenylate cyclase could be measured. For these experiments, the concentration of GTP was varied from 0.1 μM to 0.1 mM, and adenylate cyclase activity was measured in the absence and presence of the highly efficacious agonists carbachol and oxotremorine-M. It can be seen in Fig. 2 that the inhibition of adenylate cyclase activity elicited by carbachol (1.0 mM) and oxotremorine-M (0.1 mM) was maximal (52–55%) at 0.1 mM GTP and only declined to approximately 34–39% as the GTP concentration decreased to 0.1 μM.

Having established that muscarinic receptor-mediated inhibition of adenylate cyclase activity could be measured reliably at concentrations of GTP as low as 0.1 μM, we set out to determine what effect changing the concentration of GTP would have on the concentration-effect relationship of carbachol and oxotremorine-M for inhibiting adenylate cyclase activity. For these experiments, the concentration of GTP was varied from about 0.1 μM to 0.1 mM. Such a change in the concentration of GTP had a rather marked effect on the binding affinities of carbachol and oxotremorine-M (see below). In the presence of 0.1 μM GTP, the maximal effect of oxotremorine-M on adenylate cyclase activity was 38% inhibition, and the EC_{50} value of oxotremorine-M was 0.17 μM (see Fig. 3A). In the presence of 0.1 mM GTP, the maximal effect of oxotremorine-M increased to 50% and the EC_{50} value increased about 5-fold. Similar observations were made in experiments with carbachol (Fig. 3B). The effects of GTP on the concentration-effect curves of carbachol and oxotremorine-M are listed in Table 1. In assessing the effects of GTP on the concentration-effect relationship of the agonists, it is important to compare

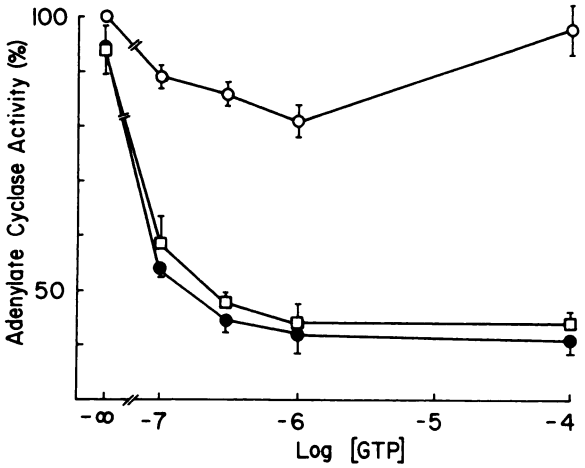

Fig. 2. Effects of GTP on muscarinic agonist-mediated inhibition of adenylate cyclase activity in the rat myocardium. Adenylate cyclase activity was measured at the indicated concentrations of GTP and in the absence (○) and presence of the highly efficacious muscarinic agonists carbachol (1.0 mM) (□) and oxotremorine-M (0.1 mM) (●). The data points represent the mean values ± standard errors of four experiments, each done in triplicate on individual rats. The data are expressed as a percentage of the adenylate cyclase activity measured in the absence of GTP and the muscarinic agonists.

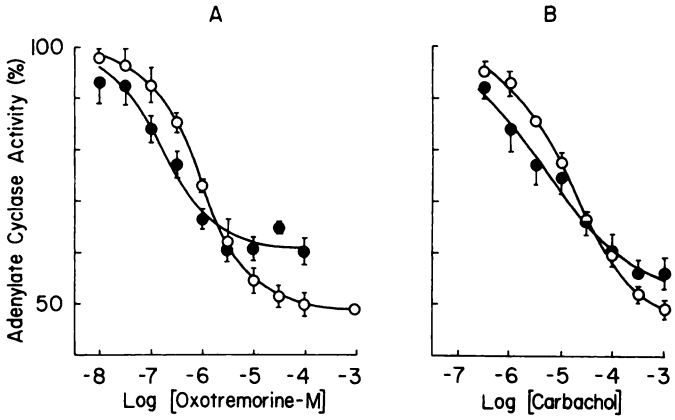

Fig. 3. Effects of GTP on the inhibition of adenylate cyclase activity elicited by various concentrations of oxotremorine-M and carbachol. Each *point* represents the mean binding value ± the standard error of five experiments, each done in triplicate. A, Adenylate cyclase activity was measured in the presence of the indicated concentrations of oxotremorine-M and in the presence of 0.1 μM GTP (●) and 0.1 mM GTP (○). B, Adenylate cyclase activity was measured in the presence of the indicated concentrations of carbachol and in the presence of 0.3 μM GTP (●) and 0.1 mM GTP (○).

TABLE 1

Effects of GTP on the inhibition of adenylate cyclase activity elicited by oxotremorine-M and carbachol

The parameters were calculated from the data shown in Fig. 3.

Agonist	GTP	EC_{50}	I_{max}	CE[a]
	μM	μM	%	
Oxotremorine-M	0.1	0.17	38	0.11[b], 0.31[c]
	100	0.91	50	1.0
Carbachol	0.3	5.3	49	0.24[b], 0.38[c]
	100	16	55	1.0

[a] The relative coupling efficiency (CE) was calculated from the plots in Fig. 5, as described in the text.

[b] The relative coupling efficiency was calculated assuming that agonist occupancy in the presence of 0.1 mM GTP was consistent with a simple one-site model having a dissociation constant equivalent to the X_{50} value of the agonist/[^{3}H]NMS competition curve.

[c] The relative coupling efficiency was calculated assuming that agonist occupancy in the presence of 0.1 mM GTP was described by the agonist/[^{3}H]NMS competition curve after correction for the competitive shift caused by [^{3}H]NMS.

equiactive agonist concentrations in the presence of low and high concentrations of GTP. This comparison showed that, for levels of enzyme inhibition up to about 40%, equiactive agonist concentrations varied less than 3-fold at the two different concentrations of GTP. Consequently, we conclude that increasing the concentration of GTP from 0.1 μM to 0.1 mM had a small to moderate effect on agonist-mediated inhibition of adenylate cyclase activity, as compared with the rather large effect on agonist binding affinity described below.

Muscarinic receptor binding properties. The effects of GTP on the binding of carbachol and oxotremorine-M were investigated by measurement of the competitive inhibition of the binding of [^{3}H]NMS by carbachol and oxotremorine-M in the presence of various concentrations of GTP. Fig. 4A shows the competitive inhibition of [^{3}H]NMS binding by oxotremorine-M in the absence and presence of various concentrations of GTP ranging from 0.1 μM to 0.1 mM. It can be seen that GTP caused a concentration-dependent decrease in the binding affinity of oxotremorine-M, so that the IC_{50} value of oxotremorine-M increased about 560-fold in the presence of 0.1 mM GTP. GTP was without effect on the binding of [^{3}H]NMS

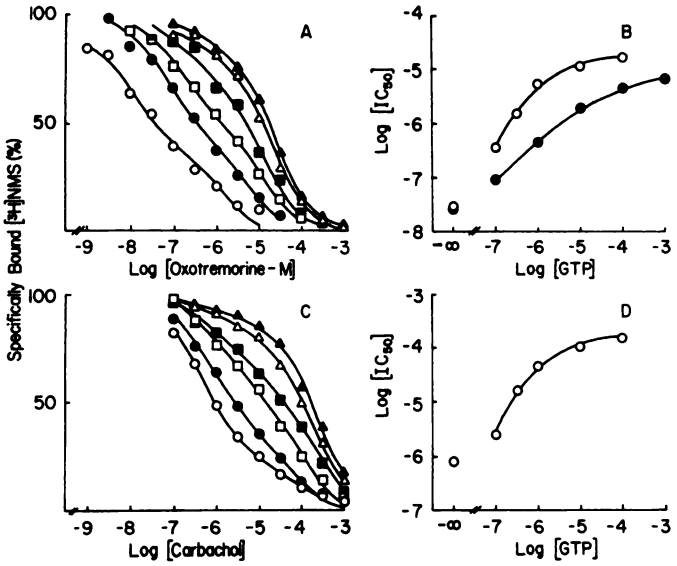

Fig. 4. Effects of GTP on the competitive inhibition of [^{3}H]NMS binding by oxotremorine-M and carbachol. Each *point* in A and C represents the mean binding value from five experiments, each done in triplicate. The average standard error of the mean was 2.7%. A, The competitive inhibition of the specific binding of [^{3}H]NMS by oxotremorine-M was measured in the absence (○) and presence of GTP at concentrations of 0.1 μM (●), 0.3 μM (□), 1.0 μM (■), 10 μM (△), and 100 μM (▲). The binding assay was carried out in a buffer identical to that of the adenylate cyclase assay. The concentration of [^{3}H]NMS was approximately 0.5 nM. B, ○, the IC_{50} values from the competition curves shown in A are plotted against the concentration of GTP; ●, the experiments shown in A were repeated in the absence of the nucleotide-regenerating system and the corresponding IC_{50} values are plotted against the concentration of GTP. C, The competitive inhibition of the specific binding of [^{3}H]NMS by carbachol was measured in the absence and presence of various concentrations of GTP, as indicated in A. D, The IC_{50} values for the competition curves shown in C are plotted against the concentration of GTP.

under the present assay conditions. These experiments were repeated in the absence of the nucleotide-regenerating system, to determine whether the regenerating system had an influence on binding properties (see Fig. 4B). In the absence of GTP, the IC_{50} values of oxotremorine-M were practically the same in both the presence (IC_{50} = 0.030 μM) and absence (IC_{50} = 0.025 μM) of the nucleotide-regenerating system, and the regenerating system had no effect on the binding of [^{3}H]NMS. Moreover, when the components of the regenerating system were tested individually for their effects on the binding of [^{3}H]NMS in the absence and presence of carbachol (3.0 μM), they were without effect. However, in the presence of GTP (0.1 μM to 0.1 mM), the nucleotide-regenerating system increased the IC_{50} values of oxotremorine-M 5–7-fold. Thus, we conclude that the effects of the nucleotide-regenerating system shown in Fig. 4B are due to its ability to maintain the concentration of GTP and that it is essential to include the nucleotide-regenerating system in the radioligand binding assay if an accurate estimate of agonist receptor occupancy under the conditions of the adenylate cyclase assay is to be made. In contrast, this requirement is not essential in the rabbit myocardium in the presence of high concentrations of GTP (0.1 mM) (12), presumably because of lower nucleotidease activity in the rabbit heart.

The oxotremorine-M/[^{3}H]NMS competition curve measured in the absence of GTP was inconsistent with a simple one-site model but could be described by a three-site model or the ternary complex model (analysis not shown). These observations are consistent with those of other investigators who have investigated the binding properties of muscarinic agonists in the mammalian heart (6, 13–15). In the presence of a maximally effective concentration of GTP (0.1 mM), the oxotremorine-M/[^{3}H]NMS competition curve was consistent with a two-site model exhibiting 14% high affinity sites. The effects of GTP on the carbachol/[^{3}H]NMS competition curve were qualitatively similar to those observed in competition experiments with oxotremorine-M (see Fig. 4, C and D). The effects of GTP on the X_{50} values of oxotremorine-M and carbachol are summarized in Table 2, and the results of the two-site analysis of the competition data obtained in the presence of 0.1 mM GTP are summarized in Table 3.

Occupancy-response relationships. In order to determine the influence of GTP on the ability of the agonist-receptor complex to inhibit adenylate cyclase activity, it is necessary to compare the occupancy curve of an agonist with its respective concentration-effect curve for inhibiting adenylate cyclase activity. Fig. 5 shows the results of such a comparison, where the functional responses to oxotremorine-M and carbachol, expressed relative to their own maximal responses, are plotted against the agonist concentration, together with their occupancy curves as determined by competition with [^{3}H]NMS. The occupancy curves have been corrected for the competitive shift caused by [^{3}H]NMS. A comparison of the plots in Fig. 5, A and B, shows that in the presence of 0.1 μM GTP the concentrations of oxotremorine-M causing equivalent fractional levels of response and receptor occupancy are approximately the same, whereas in the presence of 0.1 mM GTP the concentrations of oxotremorine-M causing equivalent fractional effects differ by about 16-fold. Similar observations were made for the effects of GTP on the occupancy-response relationship of carbachol (see Fig. 5, C and D).

Knowing both response and occupancy as a function of the

TABLE 2

Effects of GTP on the binding of oxotremorine-M and carbachol

The X_{50} values were calculated from the data shown in Fig. 4.

Agonist	GTP	X_{50}
	μM	μM
Oxotremorine-M	0.0	0.025
	0.1	0.31
	0.3	1.4
	1.0	4.6
	10	9.7
	100	15
Carbachol	0	0.67
	0.1	2.1
	0.3	14
	1.0	37
	10	90
	100	138

TABLE 3

Analysis of the binding properties of oxotremorine-M and carbachol in the presence of 0.1 mM GTP according to a two-site model

The binding parameters were estimated from the data shown in Fig. 3.

Agonist	K_H	K_L	High affinity sites
	μM	μM	%
Oxotremorine-M	0.26	20	14
Carbachol	0.27	177	11

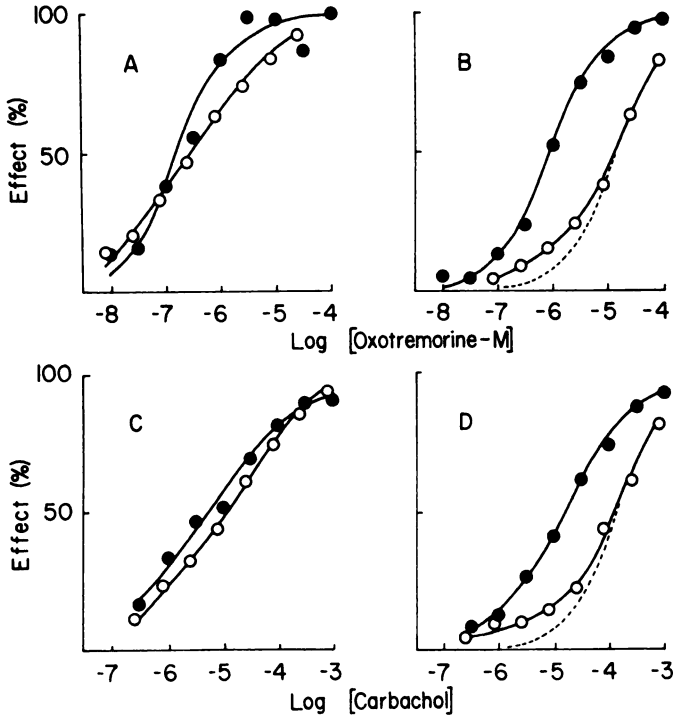

Fig. 5. Comparison of receptor occupancy (○) and inhibition of adenylate cyclase activity (●) for oxotremorine-M (A and B) and carbachol (C and D) in the presence of low (A and C) and high (B and D) concentrations of GTP. Occupancy was calculated as the percentage of specifically bound [^{3}H]NMS displaced. The occupancy values are plotted against the agonist concentration after correction for the competitive shift caused by [^{3}H]NMS. The cyclase inhibition curves have been normalized with respect to the maximum effect observed in the presence of high concentrations of the agonists. The data are from Figs. 3 and 4. The *dashed lines* in B and D designate the theoretical occupancy curve for a simple one-site model having a dissociation constant equivalent to the X_{50} value of the competition curve. The concentration of GTP was the following: A, 0.1 μM; B, 0.1 mM, C, 0.3 μM; and D, 0.1 mM.

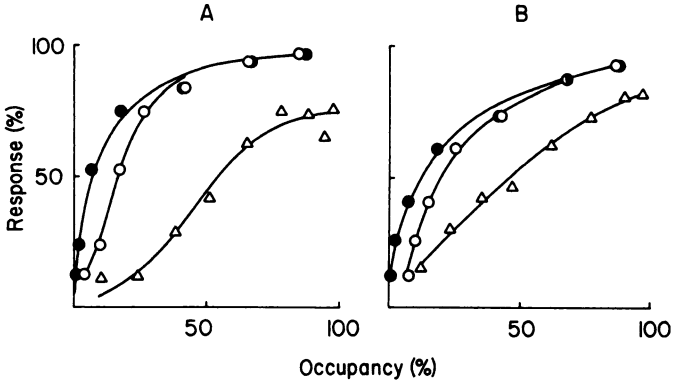

Fig. 6. Influence of a high (○, ●) and low (△) concentration of GTP on the relationship between occupancy and response for oxotremorine-M (A) and carbachol (B). The responses are expressed as a percentage of the maximum inhibition of adenylate cyclase activity elicited by oxotremorine-M in the presence of 0.1 mM GTP. ○, and △, The occupancy values were interpolated from the agonist/[^{3}H]NMS competition curve measured at a concentration of GTP equivalent to that used for measuring inhibition of adenylate cyclase activity; ●, the occupancy values were interpolated from a simple one-site model having a dissociation constant equivalent to the X_{50} value of the agonist/[^{3}H]NMS competition curve measured in the presence of 0.1 mM GTP. The plots were made from the data shown in Fig. 5. The concentration of GTP was the following: A, 0.1 mM (○, ●) and 0.1 μM (△); and B, 0.1 mM (○, ●) and 0.3 μM (△).

agonist concentration, it is possible to plot response as a function of occupancy. In Fig. 6, the responses to oxotremorine-M and carbachol are expressed relative to the maximal inhibition of adenylate cyclase activity caused by oxotremorine-M in the presence of 0.1 mM GTP. The occupancy values were calculated in two ways. For Fig. 6, *open symbols*, the occupancy values were taken directly from Fig. 5, where occupancy was calculated as the percentage of inhibition of the specific binding of [^{3}H]NMS and was plotted against the agonist concentration after correction for the competitive shift caused by [^{3}H]NMS. For Fig. 6, *closed symbols*, occupancy was calculated assuming a simple one-site model having a dissociation constant equivalent to the X_{50} value of the agonist/[^{3}H]NMS competition curve in the presence of 0.1 mM GTP. This latter method of determining occupancy was also used because 1) the agonist competition curves in the presence of 0.1 mM GTP are approximately equivalent to a simple one-site model having a dissociation constant equivalent to X_{50} and 2) a prior study showed that the X_{50} values of agonists in the presence of 0.1 mM GTP agree well with their respective dissociation constants when estimated from the adenylate cyclase data by Furchgott's method of partial receptor activation (12). It can be seen in Fig. 6 that increasing the concentration of GTP has the effect of enabling the agonist to trigger a given response at a much lower level of receptor occupancy, compared with that observed in the presence of a low concentration of GTP.

It is possible to devise a method to quantify this "apparent change in efficacy." According to classic receptor theory (16), the response to an agonist can be expressed as a function (f) of the stimulus, with the stimulus being equivalent to the product of intrinsic efficacy (ϵ) and receptor occupancy (AR):

$$\text{Response} = f(\epsilon \times AR) \quad (3)$$

In the present example, the response represents inhibition of adenylate cyclase activity and the function (f) has both amplification and transducing properties. As a point of reference, it is useful to apply Eq. 3 to the data obtained in the presence of 0.1 mM GTP. These conditions are most akin to those of the intact cell, in which the concentration of GTP is sufficient to elicit maximal effects on agonist binding and adenylate cyclase activity. When the concentration of GTP is reduced, the amplification properties of the function are diminished, so that it now requires a greater level of receptor occupancy to trigger the same response. The relationship between equivalent responses in the presence of high and low concentrations of GTP is

$$f(\epsilon \times AR) = f^*(\epsilon \times AR') \quad (4)$$

in which f^* denotes the function relating the stimulus to response in the presence of a low concentration of GTP and AR' denotes receptor occupancy in the presence of a low concentration of GTP that is required to generate a response equivalent to that of AR in the presence of 0.1 mM GTP. A useful indirect means to quantify the reduction in amplification seen at low concentrations of GTP (i.e., change from f to f^*) is to calculate the increase in receptor occupancy necessary to offset it. In other words, if it requires an increase in receptor occupancy equivalent to the ratio AR'/AR to offset the reduction in amplification caused by a decrease in the concentration of GTP, then the coupling efficiency changes by the reciprocal

amount. Thus, we define the relative coupling efficiency as:

$$CE = \frac{AR}{AR'} \quad (5)$$

in which CE denotes the coupling efficiency in the presence of a given concentration of GTP relative to that in the presence of 0.1 mM GTP. To calculate the ratio AR/AR', the level of receptor occupancy required to generate a half-maximal response in the presence of 0.1 mM GTP (AR) was divided by that required to generate an equivalent response in the presence of the lower concentration of GTP (AR'). Two different relative coupling efficiencies were calculated for each agonist, based on the two methods for calculating occupancy described above. These values are listed in Table 1. It can be seen that decreasing the concentration of GTP from 0.1 mM to 0.1 or 0.3 μM has the effect of decreasing the relative coupling efficiency of the agonists to 0.5–0.11.

Receptor modeling. In order to determine the theoretical basis for the reciprocal effects of GTP on affinity and coupling efficiency, we explored the consequences of the model described in Materials and Methods. Using Eqs. A14 and A15 in the Appendix, we calculated the theoretical occupancy curves for a highly efficacious agonist in the presence of various concentrations of GTP. The proportion of agonist bound in the form of *DRGX* was also calculated, because the rate of activation of the G protein should be proportional to the amount of agonist bound in the form of the quaternary complex (*DRGX*). The results of this analysis are shown in Fig. 7, where receptor-bound agonist in the presence of various concentrations of GTP is plotted against the agonist concentration, expressed in units relative to the agonist dissociation constant (K_1). The concentration of GTP is also indicated relative to its dissociation constant (K_3). In this example, it was assumed that the G protein concentration was 10-fold greater than the receptor concentration (i.e., $\delta = 10$). The following parameter values were used for the calculations shown in Fig. 7: $\alpha = 10^{-4}$ and $\gamma = 10^3$. The other parameter values are given in the legend to Fig. 7.

The plots in Fig. 7A show the theoretical occupancy curves for a highly efficacious agonist that has a much higher affinity for the receptor-G protein complex (RG) as compared with the free receptor (R) (i.e., $\alpha \ll 1$) and whose binding affinity is greatly reduced by GTP (i.e., $\gamma \gg 1$). The *dashed lines* in Fig. 7A indicate the proportion of drug bound in the form of the quaternary complex (*DRGX*). According to the consequences of the model, GTP causes a concentration-dependent decrease in the binding affinity of the agonist, so that in the presence of maximally effective concentrations of GTP the occupancy curve has shifted to the right 500-fold. Moreover, GTP causes an increase in both the maximum amount of quaternary complex formed and the concentration of agonist required for half-maximal formation of the quaternary complex. The calculations for the three highest concentrations of GTP have been plotted again in Fig. 7B, which shows the proportion of drug bound in the form of quaternary complex plotted on an expanded occupancy scale. Because the muscarinic receptor-adenylate cyclase system exhibits a receptor reserve, a *horizontal dashed line* has been drawn in Fig. 7B to indicate an arbitrary level of *DRGX* complex that is required to elicit a maximum inhibition of adenylate cyclase. It can be seen that the curves in Fig. 7B yield similar amounts of quaternary complex in the low agonist concentration range. Thus, at low concentrations, the agonist would trigger similar responses at the different concentrations of GTP. At high concentrations, the agonist generates greatly differing amounts of quaternary complex (*DRGX*), depending upon the concentration of GTP. Nevertheless, the amount of *DRGX* generated for the three curves is roughly equal to or greater than that required for a maximum response; consequently, similar responses would also be triggered by the agonist at high concentrations in the presence of the three different concentrations of GTP. It can be concluded from the theoretical results in Fig. 7 that the concentration-effect curve of a highly efficacious agonist is only moderately affected by a change in the concentration of GTP that causes a 10-fold reduction in agonist affinity. The effects of GTP on the theoretical curves shown in Fig. 7 are summarized in Table 4.

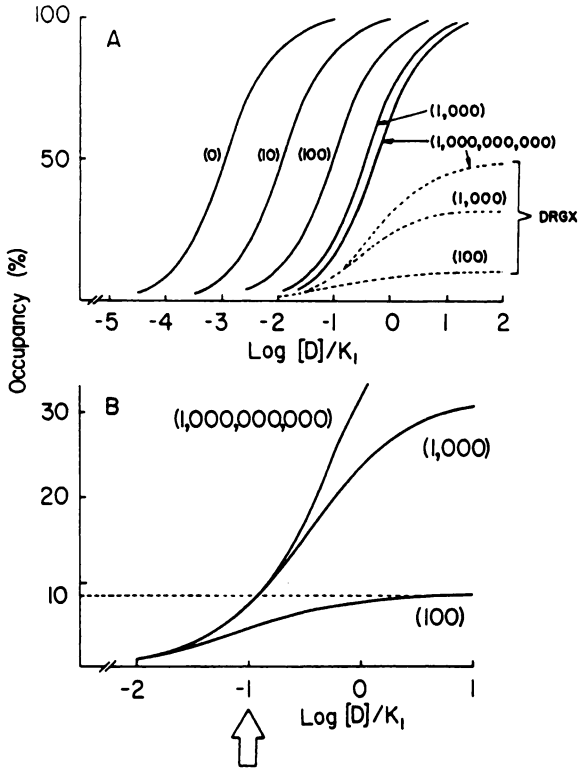

Fig. 7. Influence of GTP on agonist occupancy and the proportion of agonist bound in the form of *DRGX* to a receptor that conforms to the consequences of the ternary complex model shown in Fig. 1. The theoretical occupancy curves and the component of agonist binding attributed to the quaternary complex (*DRGX*) have been calculated using Eqs. A14 and A15 in the Appendix. For these calculations, the parameters were given the following values: $K_2 = 10^2$, $\alpha = 10^{-4}$, $\beta = 1$, $\gamma = 10^3$, and $\delta = 10$. The concentration of agonist is indicated relative to K_1 ($[D]/K_1$), and the concentration of GTP is indicated in parentheses relative to K_3 ($[GTP]/K_3$). A, Agonist receptor occupancy (*solid lines*) is plotted in the presence of different concentrations of GTP. *Dashed lines*, the component of agonist binding attributed to the quaternary complex (*DRGX*) in the presence of the three highest concentrations of GTP. B, The component of agonist binding attributed to the quaternary complex (*DRGX*) in A has been plotted again on an expanded occupancy scale for the three highest concentrations of GTP. *Horizontal dashed line*, an arbitrary level of *DRGX* required to trigger a maximum response. *Arrow*, the concentration of agonist below which the three curves yield similar amounts of quaternary complex (within a factor of 2).

A point of discrepancy between the theoretical results in Fig. 7 and the actual data for oxotremorine-M and carbachol is that the model predicts that the amount of quaternary complex

TABLE 4

Effects of GTP on the ternary complex model

The binding parameters were estimated from the theoretical data shown in Fig. 7.

[GTP]/K_3[a]	K_D/K_1[b]	K_{DRGX}/K_1[c]	$DRGX_{max}$[d]
			%
0	0.0011		0
10	0.011	0.011	0.98
100	0.10	0.10	8.3
1,000	0.40	0.40	33
1,000,000,000	0.56	0.56	49

[a] The concentration of GTP is indicated in units relative to the dissociation constant K_3.

[b] The K_D indicates the concentration of agonist required for half-maximal receptor occupancy and is expressed in units relative to the dissociation constant K_1.

[c] The K_{DRGX} indicates the concentration of agonist required for half-maximal formation of the quaternary complex (*DRGX*) and is expressed in units relative to the dissociation constant K_1.

[d] The $DRGX_{max}$ indicates the proportion of agonist bound in the form of quaternary complex (*DRGX*) and is expressed as a percentage of the total receptor concentration.

formed (*DRGX*) at any concentration of agonist is always greater at higher concentrations of GTP. This condition would lead to a greater activation of G protein and, hence, a greater inhibition of adenylate cyclase activity. Although these results were borne out at high concentrations of oxotremorine-M and carbachol, at lower concentrations of agonist receptor-mediated inhibition of adenylate cyclase activity was greater in the presence of the lower concentration of GTP (see Fig. 4). We have no adequate explanation for this discrepancy but presume that it might be accounted for by kinetic effects not addressed by our equilibrium model or that perhaps GTP has effects on G_s that complicate the picture.

A variety of other parameter values yielded results similar to those shown in Fig. 7, as long as the values of α and γ were such that the agonist induced the ternary complex ($\alpha < 1$) and GTP reduced the binding affinity ($\gamma > 1$). One such example is shown in Fig. 8, where the behavior of the model is examined under conditions similar to those described above, except that the G protein is limiting (i.e., $\delta = 0.8$ or $G_T/R_T = 0.8$). These conditions were examined because it has been shown that the behavior of agonist/[^{3}H]NMS competition curves in the heart are well described by the ternary complex model with limiting G protein (6, 15). In the absence of GTP, the agonist competition curve is complex and generally appears to exhibit high and low affinity components (see Fig. 8A). As the concentration of GTP increases, the competition curve shifts to lower affinity and is homogeneous in the presence of a maximally effective concentration of GTP. Fig. 8B shows the effects of GTP on the apparent high affinity component of the competition curve [i.e., the summation of the quaternary and ternary complexes (*DRG* and *DRGX*)]. It can be seen that GTP causes a reduction in both the apparent affinity of this component and its maximum. This high affinity component has a slope shallower than that of a simple one-site model, as indicated in Fig. 8B by the deviations between this component and the *dotted line*, which indicates the behavior of a simple one-site component. Thus, this apparent high affinity component could be described quite arbitrarily as the summation of two sites, which could explain why it has been necessary to consider a total of three sites in order to describe adequately the competition curves of muscarinic agonists in the heart (13, 14). Fig. 8D shows that, as the concentration of GTP increases, the low affinity component of binding (*DR*) increases without a change in its affinity. The effects of the three highest concentrations of GTP on the quaternary complex (*DRGX*) are shown in Fig. 8C, where the occupancy scale has been enlarged. As described in Fig. 7, an increase in the concentration of GTP causes an increase in the maximum amount of quaternary complex formed and in the concentration of agonist required for half-maximal formation of the quaternary complex. Consequently, for the reasons described above, similar amounts of the quaternary complex will be formed by the agonist at low receptor occupancy over the upper end of the concentration range of GTP. The results predict that the level of inhibition of adenylate cyclase by a highly efficacious agonist would vary only modestly as the concentration of GTP changes in this range. The effects of GTP on the various components of the agonist binding curve shown in Fig. 8 are summarized in Table 5.

Discussion

Previous studies have shown that the binding affinities of muscarinic agonists in the heart are greatly reduced by GTP (4–6) and that the concentration of a highly efficacious muscarinic agonist that yields half-maximal inhibition of adenylate cyclase activity (EC_{50}) is much less than that required for half-maximal receptor occupancy (X_{50}), provided that both parameters are measured in the presence of a high concentration of GTP (e.g., 0.1 mM) (12, 17). For less efficacious agonists, it has been shown that the binding affinity is only moderately reduced by GTP and that there is closer agreement between the concentrations of agonist that yield equivalent fractional levels of receptor occupancy and inhibition of adenylate cyclase activity (i.e., $EC_{50} \cong X_{50}$) (12, 17). In this study, we have investigated what happens to the occupancy-effect relationship when the concentration of GTP is reduced so that the binding affinity is increased about 10–50-fold. Under such conditions, we found that the concentration-effect relationships of the highly efficacious agonists oxotremorine-M and carbachol were moderately affected by the change in the concentration of GTP, so that there was closer agreement between EC_{50} and X_{50}. Thus, decreasing the concentration of GTP reduced agonist coupling efficiency and increased agonist affinity, so that there were only small to moderate effects on the concentration-effect relationship of the agonist for inhibiting adenylate cyclase activity.

Our interpretation of the data in this report rests on the assumption that the agonist/[^{3}H]NMS competition curve is an accurate measure of receptor occupancy. This assumption is supported by the results of previous studies in which agonist-mediated inhibition of adenylate cyclase activity was measured in myocardial homogenates treated with benzilylcholine mustard to inactivate a portion of the muscarinic receptors. When assays were carried out in the presence of a concentration of GTP (0.1 mM) that produced maximal effects on agonist binding, it was shown that Furchgott's method (16) yielded estimates of the dissociation constants (K_A) of agonists that were in good agreement with the X_{50} values of the agonist/[^{3}H]NMS competition curves (12). Moreover, the estimate of K_A was independent of the level of receptor inactivation, and there was good agreement between the estimates of the proportion of inactivated receptors ($1 - q$) when measured by Furchgott's method and by radioligand binding (12). These results provide strong evidence that the assumptions upon which Furchgott's method is based were met. One of these assumptions is that the agonist-receptor interaction is a bimolecular mass-action-

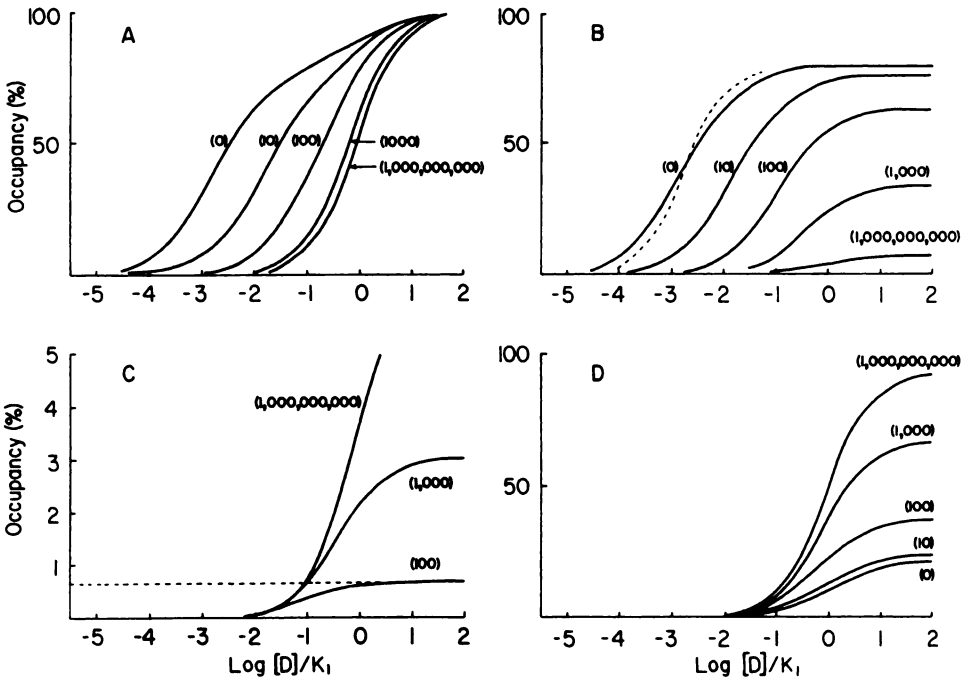

Fig. 8. Influence of GTP on the binding of an agonist to a receptor that conforms to the consequences of the ternary complex model with limiting G protein. The theoretical occupancy curves and the proportion of agonist bound in the form of the high affinity complex (*DRG* and *DRGX*), quaternary complex (*DRGX*), and low affinity complex (*DR*) were calculated using Eqs. A14–A16 in the Appendix. For these calculations, the parameters were given the following values: $K_2 = 10^2$, $\alpha = 10^{-5}$, $\beta = 1$, $\gamma = 10^4$, and $\delta = 0.8$. The concentration of agonist is indicated relative to K_1 ($[D]/K_1$), and the concentration of GTP is indicated in parentheses relative to K_3 ($[GTP]/K_3$). A, Agonist receptor occupancy is plotted in the presence of different concentrations of GTP. B, The high affinity component of agonist binding (*DRG* and *DRGX*) is plotted at various concentrations of GTP (*solid lines*). *Dashed lines*, a simple one-site model. C, The component of agonist binding that is attributed to the quaternary complex is plotted in the presence of various concentrations of GTP on an enlarged occupancy scale. *Horizontal dashed line*, an arbitrary level of *DRGX* required to trigger a maximum response. D, The low affinity component of agonist binding (*DR*) is plotted in the presence of various concentrations of GTP.

TABLE 5

Effects of GTP on the ternary complex model when the G protein is limiting

The binding parameters were estimated from the theoretical data shown in Fig. 8.

[GTP]/K_3[a]	K_D/K_1[b]	K_{DRGX}/K_1[c]	$DRGX_{max}$[d]	K_H/K_1[e]	High affinity sites[f]	K_L/K_1[g]
			%		%	
0	0.0035		0	0.0018	80	1.0
10	0.035	0.017	0.07	0.017	76	1.0
100	0.20	0.11	0.62	0.11	63	1.0
1,000	0.60	0.41	3.1	0.41	34	1.0
1,000,000,000	1.0	0.89	6.8	0.89	6.8	1.0

[a] The concentration of GTP is indicated in units relative to the dissociation constant K_3.

[b] The K_D indicates the concentration of agonist required for half-maximal receptor occupancy and is expressed in units relative to the dissociation constant K_1.

[c] The K_{DRGX} indicates the concentration of agonist required for half-maximal formation of the *DRGX* complex, expressed relative to the dissociation constant K_1.

[d] The $DRGX_{max}$ indicates the maximum amount of *DRGX* at 100% receptor occupancy, expressed as a percentage of the total receptor concentration.

[e] The K_H indicates the concentration of agonist required for half-maximal formation of the *DRGX* and *DRG* components, expressed relative to the dissociation constant K_1.

[f] The high affinity sites indicate the summation of *DRGX* and *DRG* complexes at 100% receptor occupancy, expressed as a percentage of the total receptor concentration.

[g] The K_L indicates the concentration of agonist required for half-maximal formation of the *DR* component, expressed relative to the dissociation constant K_1.

determined process. Accordingly, the results of agonist/[^{3}H]NMS competition experiments carried out under the same conditions as the adenylate cyclase assay (0.1 mM GTP) showed that the agonist competition curves were nearly consistent with a simple one-site model having an X_{50} value approximately equal to K_A. However, careful analysis of the oxotremorine-M and carbachol competition curves measured in the presence of 0.1 mM GTP (see Fig. 4 and Table 3) reveals a small population (11–14%) of relatively high affinity sites. The significance of these residual high affinity sites is unclear, and the studies summarized above suggest that they contribute very little or not at all to inhibition of adenylate cyclase. Therefore, we think that it is best to assume a simple one-site model for agonist binding in the presence of 0.1 mM GTP and that the estimates of relative coupling efficiency based on this assumption are the most accurate estimates for oxotremorine-M and carbachol (0.11 and 0.24, respectively; see Table 1).

The binding of agonists is considerably more complicated when the concentration of GTP is lowered (see Fig. 4; also see Refs. 6 and 13–15). We have attempted to use Furchgott's method to determine the K_A value of oxotremorine-M in the presence of a low concentration of GTP (0.1 μM); however, anomalous results were obtained. The estimate of K_A varied greatly, depending on the degree of receptor inactivation, and the estimate of the proportion of inactivated receptors differed greatly from that measured by radioligand binding.[2] These observations suggest that agonist binding is inconsistent with a simple one-site model in the presence of 0.1 μM GTP. Indeed, the agonist/[^{3}H]NMS competition curves measured in the presence of low concentrations of GTP (0.1–0.3 μM) were shallower than a simple one-site model, which does not contradict our conclusion that these competition curves are an accurate reflection of receptor occupancy.

In most previous studies on the mammalian heart, the analysis of agonist binding properties has relied on the superposition of independent binding sites. Using this approach, it has been clearly seen that it is essential to consider at least three types of sites in order to describe the binding properties of agonists adequately (13, 14). We also have found a significant improvement in the fit of our oxotremorine-M competition data to a

[2] F. J. Ehlert, unpublished observations.

three-site model, as compared with a two-site model (analysis not shown). An alternative approach is to analyze the binding data according to the ternary complex model. As described previously (15, 18) and in Results in connection with Fig. 8, the ternary complex model can account for binding consistent with a three-site model in the absence of GTP, and it has been shown that this model provides an excellent fit to the agonist binding data in the absence of GTP (6). However, behavior inconsistent with the ternary complex model was noted in a study on the hamster heart, where the GTP analog guanylyl 5′-imidodiphosphate only caused a small 2.3-fold reduction in the affinity of carbachol (14). We have fitted the model described in Materials and Methods (see Fig. 1) to the oxotremorine-M competitive binding curves shown in Fig. 4, using a strategy based on nonlinear regression analysis. In this analysis, we fitted Eq. A14 to the competition curves obtained in the absence and presence of various concentrations of GTP, simultaneously sharing the same parameter estimates among the curves. In so doing, we obtained a good fit to the data, with the exception that the model could not account for the residual high affinity sites in the presence of high concentrations of GTP. Consequently, we have not included the latter analysis in this report. Nevertheless, we have included a description of the ternary complex model in this report, because it is the simplest model that most closely approximates the data. The actual model is undoubtedly a heterogeneous one, which includes the ternary complex model as a major component. The heterogeneity could result from different G proteins or from something as arbitrary as damage to receptors and G proteins.

An important point with regard to the ternary complex model is that the complexity of the agonist binding curve depends on the abundance of the G protein relative to the receptor. When the G protein is in great excess relative to the receptor, the model described in the Appendix predicts that the binding of agonists should be consistent with a simple one-site model in both the absence and the presence of GTP (see Fig. 7). It is only when the G protein is limiting that the binding properties of agonists become heterogeneous in the absence of GTP (see Fig. 8). Thus, when rationalized in terms of the ternary complex model, the heterogeneous binding properties of agonists in the absence of GTP can be explained by a limiting amount of G protein ($G/R \cong 1$). However, it is known that G proteins are usually present in great excess relative to receptors in a variety of systems. This condition provides a mechanism for the amplification of the signal elicited by the binding of the agonist to the receptor and is probably a general property of G protein-linked receptors. This amplification of the signal can help to explain the large receptor reserve that exists for a variety of distal pharmacological responses to highly efficacious agonists. In an attempt to rationalize the binding properties of muscarinic agonists in terms of the ternary complex model, it has been suggested previously that perhaps G proteins have limited access to receptors in tissue homogenates or that perhaps the diffusion of the receptor and the G protein within the plane of the membrane is constrained in such a way that it cannot be described adequately by the constant K_2 (6, 15). This interpretation appears reasonable in view of a report by Haga and Haga (19) showing that, when muscarinic receptors are reconstituted in artificial membranes with an excess of G_i or G_o, agonist binding still exhibits a minor low affinity component. This low affinity component accounted for the major component of the binding in the absence of G proteins.

The results of our theoretical analysis can be rationalized on intuitive grounds. We have assumed that the pharmacological stimulus is proportional to the amount of agonist bound in the form of quaternary complex (*DRGX*) and that the amount of activated G protein is proportional to the latter. It can be reasoned further that the amount of quaternary complex formed in the presence of concentrations of GTP and agonist that saturate their respective binding sites should be greatest and that decreasing the concentration of GTP should reduce the maximum level of quaternary complex formed by the agonist at 100% occupancy. However, decreasing the concentration of GTP should also increase the affinity of agonists so that the potency of an agonist for generating the quaternary complex should be increased. Thus it might be expected that, at low levels of receptor occupancy, the amount of quaternary complex formed is constant over a range of concentrations of GTP. These predictions have been borne out by the model, as shown in Figs. 7 and 8.

In evaluating the model described in the Appendix, we investigated a variety of parameter values and have obtained results similar to those described in Fig. 7 as long as the values of the appropriate parameters were such that the propensity of the agonist to generate the ternary complex (*DRG*) is great (i.e., $\alpha K_2 \ll 1$) and that the inhibitory effects of GTP on agonist affinity is also great (i.e., $\gamma \gg 1$). These two conditions are plausible, because a variety of evidence indicates that the intrinsic efficacy of agonists is proportional to their ability to generate the ternary complex (*DRG*) and that GTP reduces the binding affinity of agonists in a manner that is proportional to efficacy (6, 17). Consequently, we feel justified in presenting the complex mathematical model with its seven parameters, because it predicts that the amount of quaternary complex formed at low receptor occupancy is constant over a range of concentrations of GTP regardless of the parameter values, except for the constraints on αK_2 and γ mentioned above.

We have evaluated the model described in the Appendix under conditions where the ratio of G protein to receptor is infinite, such that the parameter K_2 can be described by the ratio RG/R. Under these conditions, the model shown in Fig. 7 still predicts that the amount of quaternary complex formed at low receptor occupancy is constant over a range of concentrations of GTP. However, under the conditions of an infinite excess of G protein, the model predicts that all agonists, regardless of their αK_2 values, should be completely bound in the form of *DRGX* at 100% receptor occupancy in the presence of saturating amounts of GTP. Thus, under these conditions, the model cannot explain differences between full and partial agonists, because it predicts that both full and partial agonists generate the same maximum amount (i.e., 100%) of quaternary complex.

Our theoretical analysis has implications for a variety of studies in pharmacology involving G protein-linked receptors. In most cells in which the concentration of GTP has been measured, it has been found to be in the range that produces maximal effects on agonist binding (i.e., 0.1–1.0 mM; see Refs. 20–22). In the presence of excess GTP, our model predicts that the binding of agonists should be consistent with simple mass-action behavior and that the overall observed dissociation constant of an agonist (K_A) should be equivalent to the concen-

tration of agonist required for half-maximal formation of the various forms of the agonist-receptor complex (i.e., *DR*, *DRG*, and *DRGX*), with each complex being consistent with mass-action behavior. Thus, the use of classic pharmacological null methods to estimate the affinities of agonists and antagonists from functional data is justified for such model. In a recent theoretical study, Kenakin and Morgan (23) showed that the potency of an agonist for triggering a response through a G protein-linked receptor depends on the concentration of G protein in the membrane. Our model is consistent with their calculations, because it predicts that increasing the concentration of G protein would increase the amount of quaternary complex (*DRGX*) at any given concentration of agonist. This effect would increase the stimulus generated by the agonist and, thus, increase agonist potency for triggering a response. It is important to emphasize that, for a plausible model of receptor function, GTP must be present at high concentrations and have a strong inhibitory effect on agonist binding. Under such conditions, our model predicts that the overall observed dissociation constant of an agonist (K_A) is independent of the G protein concentration and is approximately equal to the dissociation constant K_1. Thus, although G proteins are likely to alter the potency of agonists for triggering a response, it would seem unlikely that the G protein concentration would influence the K_A values of agonists to any great extent in an intact cell containing physiological concentrations of GTP.

The model described in this report may have direct application in the interpretation of pharmacological experiments under conditions where the concentration of GTP is decreased. Typically, pharmacological experiments are carried out on isolated tissues that have their normal circulation interrupted and, thus, the concentration of GTP inside the tissue may fall due to a certain degree of anoxia. Under such conditions, our model predicts that the affinity and coupling efficiency of a highly efficacious agonist would change reciprocally so that the sensitivity of the tissue to the agonist would be maintained to an extent.

Our model strongly indicates that it is unlikely that GTP has any regulatory role in signal transduction, because it predicts that the flow of information through G protein-linked receptor systems is independent of GTP over a range of concentrations when the agonist is a highly efficacious hormone or neurotransmitter. Whatever the significance of the buffering effect that we describe in this report, it is interesting to note that nature has put to work a signaling pathway for hormonal stimuli that is not perturbed by a considerable variation in one of its essential cofactors, GTP.

Appendix

Equations, below, are derived that describe the binding of an agonist (D) to a receptor (R) that can associate with a G protein (G) and bind GTP (X) according to the scheme shown in Fig. 1. The dissociation constants of D for the various receptor complexes (R, RG, and RGX) are:

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

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

$$\alpha\gamma K_1 = [D][RGX]/[DRGX] \quad \text{(A3)}$$

The dissociation constants of GTP (X) for the various complexes of the G protein are given by:

$$K_3 = [G][X]/[GX] \quad \text{(A4)}$$

$$\beta K_3 = [RG][X]/[RGX] \quad \text{(A5)}$$

$$\beta\gamma K_3 = [DRG][X]/[DRGX] \quad \text{(A6)}$$

Because the equilibrium between R and G 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 dissociation constants by multiplying them by the total receptor concentration ($[R_T]$).

$$K_2 = [R][G]/[R_T][RG] \quad \text{(A7)}$$

$$\alpha K_2 = [DR][G]/[R_T][DRG] \quad \text{(A8)}$$

$$\beta K_2 = [R][GX]/[R_T][RGX] \quad \text{(A9)}$$

$$\alpha\beta\gamma K_2 = [DR][GX]/[R_T][DRGX] \quad \text{(A10)}$$

The following conservation of mass equations apply:

$$[R_T] = [R] + [DR] + [DRG] + [DRGX] + [RG] + [RGX] \quad \text{(A11)}$$

$$[G_T] = [G_B] + [G_F] \quad \text{(A12)}$$

where G_T, G_B, and G_F represent total, bound, and free G protein, respectively. The following substitution is useful:

$$\delta = [G_T]/[R_T] \quad \text{(A13)}$$

The analytical solution to this system of equations is:

$$\frac{[DR] + [DRG] + [DRGX]}{[R_T]} = \frac{[D]}{[D] + K_D} \quad \text{(A14)}$$

where:

$$K_D = \frac{1 + \frac{G_F}{K_2}\left(1 + \frac{[X]}{\beta K_3}\right)}{\frac{1}{K_1} + \frac{G_F}{\alpha K_1 K_2}\left(1 + \frac{[X]}{\beta\gamma K_3}\right)}$$

$$[G_F] = \frac{B + (B^2 + 4AC)^{1/2}}{2A}$$

$$A = 1 + \frac{[X]}{K_3}$$

$$B = \delta - 1 - K_G A$$

$$C = \delta K_G$$

$$K_G = \frac{1 + \frac{[D]}{K_1}}{\frac{1}{K_2} + \frac{[D]}{\alpha K_1 K_2} + \frac{[X]}{\beta K_2 K_3} + \frac{[D][X]}{\alpha\beta\gamma K_1 K_2 K_3}}$$

The equation describing the proportion of drug bound in the form of quaternary complex is:

$$\frac{[DRGX]}{[R_T]} = \frac{[D]}{[D] + K_{DRGX}} \quad \text{(A15)}$$

where:

$$K_{DRGX} = \frac{1 + \frac{[D]}{K_1} + \frac{G_F}{K_2} + \frac{[D]G_F}{\alpha K_1 K_2} + \frac{[X]G_F}{\beta K_2 K_3}}{\frac{[X]G_F}{\alpha\beta\gamma K_1 K_2 K_3}}$$

In this equation, G_F is defined as it is in Eq. A14. The equation describing the amount of drug bound in the form of ternary complex is:

$$\frac{[DRG]}{[R_T]} = \frac{[D]}{[D] + K_{DRG}} \quad \text{(A16)}$$

where:

$$K_{DRG} = \frac{1 + \frac{[D]}{K_1} + \frac{G_F}{K_2} + \frac{[X]G_F}{\beta K_2 K_3} + \frac{[D][X]G_F}{\alpha\beta\gamma K_1 K_2 K_3}}{\frac{G_F}{\alpha K_1 K_2}}$$

In this equation, G_F is defined as it is in Eq. A14. The amount of drug bound in the form *DR* was calculated by subtracting the summation of Eqs. A15 and A16 from Eq. A14. The concentrations of drug causing half-maximal formation of *DRGX*, *DRG*, and *DR* were estimated graphically from the plots in Figs. 7 and 8.

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