Two-state Models and the Analysis of the Allosteric Effect of Gallamine at the M2 Muscarinic Receptor

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Abbreviations: BM5, N-methyl-N-(1-methyl-4-pyrrolidino-2-butynyl) acetamide; 4-DAMP mustard, N-(2-chloroethyl)-4-piperidinyl diphenylacetate; DMEM, Dulbecco’s Modified Eagle Medium; EC50, concentration of agonist eliciting half-maximal response; Emax, maximal response, KRB, Krebs Ringer Bicarbonate; McN-A-343, [4-[[N-(3-chlorophenyl)carbamoyl]oxy]-2-butynyl]trimethylammonium; NMS, N-methylscopolamine
ABSTRACT

We measured the influence of gallamine on the functional responses and binding properties of selected agonists at the M$_2$ muscarinic receptor and analyzed the data within the context of the allosteric ternary complex model. Our analysis showed that gallamine modified agonist affinity without influencing efficacy. To explain this behavior, we investigated the allosteric ternary complex model at a deeper level of analysis to assess allostery in terms of the differential affinity of gallamine for ground and active states of the receptor. Our simulations showed that two-state models based on a single orthosteric site for the agonist linked to an allosteric site for gallamine could not account for affinity-only modulation, even if multiple conformations of ground and active states were considered. We also expanded the tandem two-site model of Jakubik et al. (J. Biol. Chem. 275:18836-18844, 2000) within the context of the allosteric ternary complex model and analyzed the resulting hybrid model at the level of receptor states. This model posits that the agonist first binds to a relay site and then shuttles to the activation site to turn on the receptor. If it is assumed that allostery occurs at the relay site and not the activation site, then this model can account for affinity-only modulation in a manner consistent with the allosteric ternary complex model.
INTRODUCTION

A variety of drugs have been shown to modulate the binding of ligands allosterically to the primary recognition site (orthosteric site) of muscarinic receptors (Stockton et al., 1983; Birdsall and Lazareno, 2005). Allosterism is often analyzed within the context of the allosteric ternary complex model shown in Figure 1b, which illustrates that both orthosteric and allosteric ligands bind to their respective sites on the same receptor with dissociation constants of $K_X$ and $K_A$, respectively (Stockton et al., 1983; Ehlert, 1988a). When both ligands are bound to the receptor, their observed dissociation constants ($K_{obs}$) are modified by the factor $\alpha$, which is a measure of the cooperativity between the binding of the two ligands. By considering that the ternary complex ($XRA$) might have an altered intrinsic efficacy ($\epsilon'$) compared to that of the binary ($XR$) complex ($\epsilon$), it is possible to measure allosteric modulation of intrinsic efficacy in functional experiments (Ehlert, 1988a; Ehlert, 2005).

We can also consider the allosteric model at a deeper level of analysis and examine how the allosteric ligand changes the affinity and intrinsic efficacy of the orthosteric ligand-receptor complex. The two-state allosteric model described mathematically in Figure 1c and schematically in Figure 2a is the simplest way to address this question. If the orthosteric and allosteric ligands exhibit the same preference for the ground and active states, then the interaction is positively cooperative ($\alpha > 1$), whereas if the ligands exhibit the opposite selectivity then the interaction is negatively cooperative ($0 < \alpha < 1$). Predictions from this model include a correlation between the quality (negative or positive) and magnitude of the cooperativity and the intrinsic efficacy of the orthosteric ligand-receptor complex. Also, if the orthosteric ligand lacks sufficient intrinsic efficacy to activate the receptor completely at 100% occupancy in both the absence and presence of the allosteric modulator, then the modulation in affinity occurs with a simultaneous modulation in the proportion of receptors in the active state at 100% receptor occupancy (i.e., efficacy modulation). Heteromeric GABA$_A$ receptor subtypes
exhibit many of the predictions of the two-state allosteric model with regard to benzodiazepines and other allosteric modulators (Ehlert et al., 1983; Levitan et al., 1988; Sigel and Baur, 1988).

The neuromuscular blocking agent gallamine has been shown to inhibit both functional responses and binding properties of orthosteric muscarinic ligands at the M₂ muscarinic receptor allosterically (Clark and Mitchelson, 1976; Stockton et al., 1983; Ehlert, 1988b; Lazareno and Birdsall, 1995). So far, only parallel shifts have been reported for the effect of gallamine on the concentration-response curves of agonists including the partial agonist BM5, suggesting that gallamine modifies the affinity of agonists without influencing their intrinsic efficacy. In contrast, strichnyne (Lazareno and Birdsall, 1995) and alcuronium (Zahn et al., 2002) have been shown to reduce both the potency and maximal responses of muscarinic agonists through an allosteric mechanism. It has been shown that the allosteric shift in the concentration-response curve of a full agonist for eliciting a downstream response is equivalent to the product of the change in affinity ($p$, equation 9) and efficacy ($q$, equation 10) caused by the modulator (Ehlert, 1988a; Ehlert, 2005). When the modulator is present at a maximally effective concentration, these changes in observed affinity and intrinsic efficacy are equivalent to the cooperativity constants $\alpha$ and $\beta$ (see Appendix). If gallamine only modulates the affinity of muscarinic agonists, then its effect on the potency of an agonist for eliciting a response should be equivalent to its effect on binding affinity.

In the present report, we have tested this postulate and found that gallamine only modulates the affinity of a group of agonists. We show that this behavior is inconsistent with a simple two-state allosteric model (Figure 1c and 2a) as well as a more complex one exhibiting two ground and two active conformations of the receptor (Figure 2b). Since studies on mutagenesis (Matsui et al., 1995; Hulme et al., 2003) and the kinetics of ligand binding (Jakubik et al., 2000) suggest the presence of an accessory site on the muscarinic receptor that relays the orthosteric ligand to the primary activation site, we have considered allosterism based on the tandem two-site model of Jakubik (2000) (Figure 2c). We show that this model can account for the selective modulation of agonist affinity by gallamine if it is assumed that allosterism occurs...
at the relay site and not the activation site. Although the three models examined (Figure 2) differ at the level of receptor states, they are all consistent at the level of receptor complexes, illustrating the usefulness of the simple allosteric model (Figure 1b) in quantifying allosteric modulation.
METHODS

Cell culture: Chinese hamster ovary cells stably expressing the human M₂ muscarinic receptor (CHO hM₂ cells) were obtained from Acadia Pharmaceuticals (San Diego, CA) and cultured in Dulbecco’s Modified Eagle Medium, with high glucose plus L-glutamine, supplemented with 10% fetal calf serum, sodium bicarbonate (3.7 g/L), and penicillin-streptomycin (100 units/ml) (DMEM) containing G 418 (0.3 mg/ml) at 37°C in a humidified atmosphere with 5% CO₂.

Cyclic AMP accumulation: Muscarinic agonist mediated inhibition of forskolin-stimulated cAMP accumulation was measured in CHO hM₂ cells using a modification of the [³H]adenine prelabeling method of Schultz et al. (1972) and the chromatography procedure of Salomon et al. (1974). Confluent cell monolayers grown in T75 flasks were washed with DMEM media and then incubated in 9 ml DMEM containing [³H]adenine (60 µCi) and adenine (3 µM) for one hour at 37°C in 5% CO₂. Cells were washed twice with DMEM and harvested using trypsin. The resulting intact cell suspensions were centrifuged for 10 minutes at 350 g, suspended in Krebs Ringer Bicarbonate buffer (KRB buffer; 124 mM NaCl, 5 mM KCl, 1.3 mM MgCl₂, 26 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.8 mM CaCl₂, and 10 mM glucose) at pH 7.4 and centrifuged a second time. Cells were suspended in KRB buffer containing isobutylmethylxanthine (0.5 mM) and incubated for an additional 10 min at 37°C prior to use in the cAMP assay. Muscarinic agonist mediated inhibition of cAMP accumulation was carried out in plastic tubes containing intact cells, forskolin (10 µM), isobutylmethylxanthine (0.5 mM) and various concentrations of muscarinic agonist in a final volume of 0.35 ml of KRB buffer. Tubes were incubated at 37°C for 12 min in a water bath. The reaction was started by the addition of cells and was stopped by addition of an aliquot (0.2 ml) of ice-cold 30% (w/v) trichloroacetic acid. After at least 30 minutes on ice, the tubes were centrifuged for 10 min at 3,000 g, and an aliquot (0.5 ml) from each tube was applied to a column of 1.5 ml Dowex (AG-50W-X4, 200-400 mesh) and washed with two aliquots of water (1.25 ml each) to remove [³H]ATP. The [³H]cAMP was eluted onto a
column of neutral alumina (0.6 g) with 4 ml of water and then eluted into scintillation vials with 4 ml of 0.1 M imidazole HCl (pH 7.5). The samples were counted by liquid scintillation spectroscopy.

**Intact cell binding assay:** Muscarinic receptor binding was measured using [³H]N-methylscopolamine ([³H]NMS, 80 Ci/mmol, Dupont NEN, Boston, Massachusetts). CHO hM₂ cells were harvested as described above for the cAMP assay. Cells were suspended in an appropriate amount of KRB buffer such that specific [³H]NMS binding never exceeded 20% of the total amount of added [³H]NMS. For agonist/[³H]NMS competitive binding assays measured in the absence and presence of gallamine (10 µM), cell suspensions were incubated for 15 min at 37°C in 1.0 ml of KRB buffer containing [³H]NMS (1.0 nM) and various concentrations of agonist. Preliminary experiments in the absence and presence of gallamine (10 µM) showed that the competition curves were unaffected by a change in the incubation time from 10 to 30 min; consequently, we chose 15 min as our standard incubation time for these experiments. In some experiments with arecoline, we measured binding in the presence of 100 µM gallamine. For these experiments we extended the incubation to 90 min (the control competition curve was also incubated for 90 min), because we found that a shorter incubation underestimated the effect of gallamine whereas a longer, three-hr incubation yielded results similar to the 90 min incubation. A two-hr incubation was used in experiments investigating the influence of various concentrations of gallamine on the binding of [³H]NMS. The incubation with [³H]NMS was always started by the addition of cells. All assays were run in triplicate, and nonspecific binding was defined as the residual binding in the presence of 10 µM atropine. Specific [³H]NMS binding was trapped by rapid filtration on Whatman glass fiber filters (G/FB) using a cell harvester (Brandel, Gaithersberg, MD). The filters were rinsed with 3 aliquots (4 ml each) of ice-cold saline and counted.
Analysis of biological data: The observed dissociation constant of \[^{3}H\]NMS (\(K_{\text{obs-NMS}}\)) was estimated from the results of equilibrium measurements of the specific binding (\(B\)) at various concentrations of the radioligand. To estimate \(K_{\text{obs-NMS}}\), the following equation was fitted to the data by nonlinear regression analysis:

\[
B = \frac{XB_{\text{max}}}{X + K_{\text{obs-NMS}}}
\]

in which \(X\) denotes the concentration of \[^{3}H\]NMS. The data for the inhibition of the binding of a fixed concentration of \[^{3}H\]NMS by various concentration of gallamine (\(A\)) were fitted to the following equation by nonlinear regression analysis (Ehlert, 1988a):

\[
B = Y_{o} \left( \frac{1 - Y'}{1 + \frac{A}{A_{50}}} + Y' \right)
\]

in which \(Y_{o}\) denotes the maximal amount of \[^{3}H\]NMS bound in the absence of gallamine, \(Y'\) denotes the fractional estimate of the residual \[^{3}H\]NMS bound at maximally effective concentration of gallamine, and \(A_{50}\) denotes the concentration of gallamine causing half of its maximal effect. As described previously (Ehlert, 1988a), the estimate of the cooperativity (\(\alpha\)) for the interaction between gallamine and \[^{3}H\]NMS can be calculated from the following equation:

\[
\alpha_{\text{NMS}} = \frac{Y'}{1 + AK_{1-NMS}(1 - Y')}
\]

in which \(K_{1-NMS}\) denotes the affinity constant of \[^{3}H\]NMS for its site on the muscarinic receptor in the absence of gallamine. The constant \(K_{1}\) has units of inverse molarity and is equivalent to the reciprocal of \(K_{\text{obs-NMS}}\) value measured in the absence of gallamine. In equation 3, a value of \(\alpha\) greater than one denotes positive cooperativity, whereas a value between 1 and 0 denotes negative cooperativity. Thus, the definition of \(\alpha\) in this study is equivalent to the reciprocal of that described in Ehlert (1988a). The estimate of the affinity constant of gallamine for it binding site in the absence of NMS (\(K_{2}\)) can be calculated as described previously:

\[
K_{2} = \frac{XXK_{1-NMS} + 1}{(X\alpha_{\text{NMS}}K_{1-NMS} + 1)A_{50}}
\]
As described above for $K_1$, $K_2$ also has units of inverse molarity. The agonist/[³H]NMS competition curves were analyzed by nonlinear regression analysis according to the following equation to estimate the concentration of agonist causing half-maximal inhibition of specific [³H]NMS binding ($IC_{50}$):

$$B = Y_o \left( \frac{1}{1 + \frac{D^H}{IC_{50}^H}} \right)$$

in which $D$ denotes the concentration of agonist, and $H$ denotes the Hill slope. For those experiments in which the agonist/[³H]NMS competition curve was measured in the absence of gallamine, the $IC_{50}$ value was corrected for the competitive effect of NMS to yield the affinity constant for the agonist ($K_{1-D}$) (Cheng and Prusoff, 1973):

$$K_{1-D} = \frac{1 + K_{1-NMS}[[³H]NMS]}{IC_{50}}$$

The cooperativity between the binding of agonist and gallamine ($\alpha_D$) was estimated from the agonist/[³H]NMS competition curve measured in the presence of gallamine using the following equation, which is derived from equation 54 under “Appendix”:

$$\alpha_D = \frac{1}{R} \left( \frac{1 - R}{AK_2} + \frac{1 + \alpha_{NMS}[[³H]NMS]K_{1-NMS}}{1 + [[³H]NMS]K_{1-NMS}} \right)$$

In this equation $R$ denotes the ratio of the $IC_{50}$ value of the agonist measured in the presence of gallamine divided by that measured in its absence.

**Drug and chemicals:** Drugs and chemicals were obtained from the following sources: [³H]adenine and [³H]NMS, PerkinElmer Life and Analytical Sciences, Boston, MA; DMEM and trypsin-EDTA, Invitrogen, Carlsbad, CA; acetylcholine, atropine, carbachol and gallamine, Sigma Chemical Company, St Louis, MO; oxotremorine-M, Sigma RBI, Natick, MA. The compound 4-DAMP mustard (N-(2-chloroethyl)-4-piperidinyl diphenylacetate) was synthesized as described previously (Thomas et al., 1992).
RESULTS

Biological assays

**Influence of gallamine on the binding of [³H]NMS:** The influence of a single concentration of gallamine (10 µM) on the equilibrium binding of [³H]NMS in intact CHO cells stably expressing the human M₂ muscarinic receptor is shown in Figure 3a. In the absence of gallamine, the [³H]NMS saturation curve was consistent with a simple one-site model having a binding capacity of 64 fmol per assay and an observed dissociation constant of 0.144 nM (1/K₁). In the presence of gallamine, the observed dissociation constant of [³H]NMS increased 8.3-fold to 1.20 nM without a significant effect on binding capacity (F₁,₄₄ = 1.5; P = 0.23). We also measured the influence of various concentrations of gallamine on the binding of [³H]NMS at a fixed concentration (see Figure 3b). Gallamine caused a concentration-dependent inhibition of [³H]NMS binding with the IC₅₀ for this effect being 1.97 µM. Over the concentration range investigated, gallamine did not fully displace specific [³H]NMS binding, but rather the binding reached a plateau of 21% at high concentrations of gallamine. Knowing this plateau value and the equilibrium dissociation constant of [³H]NMS, it is possible to estimate a cooperativity value α of 0.060 for the interaction between [³H]NMS and gallamine using equation 3. It is also possible to estimate the dissociation constant of gallamine in the absence of [³H]NMS using equation 4 and the estimates of K₁ and α. All of these parameter estimates are listed in Table 1. These results are consistent with the findings of others (Stockton et al., 1983).

**Influence of gallamine on the binding of agonists:** We also investigated the influence of gallamine on the binding affinities of the muscarinic agonists, acetylcholine, S-aceclidine, carbachol and oxotremorine-M, in CHO cells stably expressing the human M₂ muscarinic receptor. For these experiments we measured the competitive inhibition of the binding of [³H]NMS at a fixed concentration (approximately 1 nM) by increasing concentrations of the
agonists. We also repeated these experiments in the presence of a fixed concentration of
gallamine (10 µM). The results of these experiments are shown in Figure 4 where the agonist
curves have been scaled to the maximal binding observed in the absence of agonist. This
maximal binding value was inhibited approximately 64% in the presence of gallamine (10 µM).
The competition curves were consistent with a simple one-site model having a Hill slope of
approximately one. Gallamine shifted the agonist/[3H]NMS competition curves to the right about
four-fold for each agonist. The IC$_{50}$ values and Hill slopes for these competition curves are listed
in Table 2. The IC$_{50}$ value measured in the absence of gallamine was corrected for the
competitive effect of [3H]NMS using equation 6 to yield the true affinity constants of the
agonists, and these estimates are listed in Table 1. Knowing the shift in the competition curve
caused by gallamine as well as the binding parameters of [3H]NMS and gallamine (see Table 1),
it is possible to estimate the cooperativity constant (α) for the allosteric interaction between
gallamine and the agonists using equation 7. These cooperativity estimates are listed in Table 1.
Compared to [3H]NMS, which exhibited a 17-fold reduction in affinity for the gallamine
occupied receptor compared to the free receptor, the corresponding reduction in affinity for
agonists was approximately 100-fold.

We also investigated the influence of gallamine at three concentrations (3, 10 and 100
µM) on the binding of arecoline (Figure 4e). For the data obtained in the presence of 100 µM
gallamine as well as a paired set of control binding measurements, we extended the incubation
time to 90 min to ensure that equilibrium was achieved as explained under “Methods.” The data
were calculated as described in the previous paragraph. The IC$_{50}$ values of the control inhibition
curve and that measured in the presence of 10 µM gallamine are listed in Table 2, and the
average estimates of $K_f$ and cooperativity constant (α) for the interaction between gallamine and
arecoline are listed in Table 1.

Influence of gallamine on the functional response of agonists: We also investigated the
influence of gallamine (10 µM) on the functional responses to the same muscarinic agonists in
CHO cells stably transfected with the human M₂ muscarinic receptor. In these assays we measured agonist-mediated inhibition of forskolin stimulated cAMP accumulation (Figure 5) under conditions that were nearly identical to those of the binding assay. Each agonist caused a concentration-dependent inhibition of cAMP accumulation, with the maximal effect reaching a plateau at approximately 65% inhibition. In the presence of gallamine (10 µM), the inhibition curves shifted to the right approximately 14-fold, without a significant effect on the maximal inhibition (P values of 0.40, 0.92, 0.95, 0.74 and 0.22 for the lack of effect of gallamine on the $E_{\text{max}}$ values of acetylcholine, S-aceclidine, arecoline, carbachol, and oxotremorine-M, respectively, (paired T test)). We also investigated two additional concentrations of gallamine (3 µM and 100 µM) on the inhibition of cAMP accumulation elicited by arecoline. A summary of the influence of gallamine on the $EC_{50}$ and $E_{\text{max}}$ values of the agonists is given in Table 3.

It is possible that an allosteric mediated reduction in agonist efficacy could be manifest without a change in the $E_{\text{max}}$ of the agonist, if the agonist is highly efficacious and elicits a maximal response at a submaximal level of receptor occupancy. To explore this issue, we treated CHO cells with cyclized 4-DAMP mustard (40 nM for 30 min; see Thomas et al. (1992)) to inactivate a portion of the M₂ muscarinic receptor (Figure 5f). After washing the cells, we measured oxotremorine-M-mediated inhibition of forskolin stimulated cAMP accumulation in control and 4-DAMP mustard treated cells. Treatment with 4-DAMP mustard caused a reduction in the potency of oxotremorine-M (control, $pEC_{50} = 6.67$; 4-DAMP mustard-treated, $pEC_{50} = 6.05$) and a decrease in the maximal response to 38% inhibition of cAMP accumulation (control $E_{\text{max}} = 56\%$). Analysis of these data by the operational model yielded estimates of the negative logarithm of the dissociation constant of oxotremorine-M ($pK_{\text{obs}} = 6.03 \pm 0.27$) and the proportion of residual receptors not inactivated by 4-DAMP mustard (0.3 \pm 0.09) (Black and Leff, 1983). We also measured the effects of gallamine (10 and 100 µM) on the response to oxotremorine-M after 4-DAMP mustard treatment, and these results are summarized in Table 3. Gallamine caused parallel dextral shifts in the concentration-response curves of oxotremorine-M.
without affecting the maximal response (P values of 0.97 and 0.45 for the lack of effect of 10 and 100 µM gallamine on the $E_{\text{max}}$, respectively).

Comparison of the effects of gallamine in binding and functional assays: The influence of gallamine on the response to an agonist can be attributed to a change in the affinity or the intrinsic efficacy of the agonist-receptor complex or some combination thereof. For a highly efficacious agonist exhibiting a receptor reserve for the response at EC$_{50}$, the allostERIC shift in the agonist concentration-response curve is equivalent to the product of the affinity and intrinsic efficacy of the agonist in the presence of the modulator expressed relative to that measured in the absence of the modulator (see Ehlert (2005)). Thus, by comparing the total effect of gallamine in a functional assay (change in affinity and efficacy) with its effect on observed affinity in a binding assay (change in affinity only), it should be possible to dissect out its modulatory effects on the affinity and intrinsic efficacy components of the agonist-receptor complex. When used at a concentration of 10 µM, gallamine caused shifts of approximately 14-fold in the concentration response curves of the various agonists. Knowing the true affinity constant ($K_I$) of each agonist as well as its cooperativity constant ($\alpha$), it is possible to estimate the reduction in the affinity of the agonist-receptor complex caused by gallamine using equation 18 under “Appendix” and the corresponding parameters estimated in binding assays. These calculations yielded estimates of approximately 15-fold for the reduction in affinity of the agonist-receptor complex caused by gallamine at 10 µM. We also calculated the theoretical shifts for the other concentrations of gallamine used functional assays with arecoline and oxotremorine-M. These estimates of the predicted reduction in affinity are listed in Table 3 together with the observed shift in the agonist concentration-response curve. The close agreement between the two estimates shows that the modulatory effect of gallamine on the activity of the agonists can be attributed entirely to a reduction in affinity.
Mathematical modeling

To understand the mechanism for the selective modulation in agonist affinity by gallamine, we explored three distinct allosteric models of two general types. The first type represents a standard two-state model having ground and active states of the receptor with orthosteric and allosteric binding sites, in which the degree of receptor activation is proportional to the amount of receptor in the active state. Within this context, we explored two subtypes of models, the first having only one conformation of each state (simple two-state model) and the second having two ground and two active conformations (complex two-state model). The second overall type of model that we explored was the tandem two-site model of Jakubik et al. (2000). This model posits that the orthosteric ligand first binds upon the receptor at a relay site and then shuttles to the activation site of the receptor. Both sites contribute to the overall affinity of the orthosteric ligand, but only the activation site triggers the stimulus. This model introduces the possibility that allosteric regulation can occur at the relay site, the activation site or both. In our analysis, we explored the allosteric regulation at the relay site only, because such a mechanism can easily explain why only the affinity of orthosteric agonists is modulated by gallamine and not intrinsic efficacy. We considered that both the relay site and the activation site exist in two conformations. The two conformations of the relay site contribute to affinity only, whereas the two conformations of the activation site represent ground and active states. Thus, these states contribute to both affinity and intrinsic efficacy.

Our modeling addresses three hierarchical levels of analysis. At the first level all three models are equivalent and can be described by a simple one-site model having an observed dissociation constant ($K_{obs}$) that we express in units of molarity (see Figure 1a). At the second level, all three models are also equivalent, and can be described by the simple allosteric ternary complex model shown in Figure 1b. At this level of analysis, we use affinity constants with inverse molarity units and cooperativity constants (e.g., $\alpha$) to describe the microscopic binding affinities of the binary ($XR$, $RA$) and ternary ($XRA$) complexes. At both the first and second
levels, the tandem two-site model is more complicated than the state models, yet it still reduces to the simple allosteric ternary complex model (see Discussion and Appendix, equations 52 and 53). At the third level of analysis, the three models are distinct and are characterized by a unique set of microscopic affinity constants for different conformations of the receptor (Figures 1c, 8c and 10c). These are expressed in inverse molarity units. We do not use cooperativity constants at this level of analysis because each conformation represents a unique structure.

In most pharmacological experiments, it is only possible to estimate the second level parameters (i.e., the observed affinity of the orthosteric \( K_1 \) and allosteric \( K_2 \) ligands and the maximal change in affinity (\( \alpha \)) and efficacy (\( \beta \)) caused by the allosteric modulator) and not the microscopic affinity constants of the various states of the receptor (level three parameters). The goal of this analysis is to show how the second level parameters are related to the level three parameters and how, in some instances, it is possible to estimate the ratio of microscopic affinity constants of the allosteric modulator for different states of the receptor (e.g., \( K_f/K_e \), two state models).

Our conformational analysis (level three) is distinct from the analysis described by Hall (2000). In the latter analysis ground and active states of the receptor were considered, yet the affinity of each state was under allosteric regulation and could exhibit a range of observed affinities. This behavior can only occur if each so-called state represents an equilibrium among additional, undefined conformations with unique affinity constants. Thus, it is impossible to determine whether some of the cooperativity values explored by Hall (2000) are actually possible within the constraints of a two-state model. In other words, to explore the consequences of an allosteric model, one cannot simply assign different values to the cooperativity constants and see how the model behaves. Rather, one needs to assign different values to the microscopic constants that determine the cooperativity constants to explore the permissible range of cooperativity constants. In this regard, we follow the convention for defining microscopic constants established by Monod, Wyman and Changeux (1965) and others (Koshland et al.,
1966; Colquhoun and Sakmann, 1985) and designate each conformation as unique with a distinct, unvarying affinity constant.

**Simple two-state model:** Figure 1 illustrates how the allosteric model can be expanded to incorporate ground and active states of a receptor. The resulting model is a simplified form of that described by Hall (2000), and its derivation has been described within the context of the ternary complex model (Ehlert, 2000). Each side of the allosteric model (Figure 1b) has been expanded into a square representing the equilibrium between inactive and active states of the receptor. Each square represents a vertical side of the cube shown in Figure 1c. The upper horizontal square of the cube represents all receptor complexes in the active state, whereas the lower horizontal square denotes those of the inactive state. The inactive and active states each have unique microscopic affinity constants for the orthosteric ($K_a$ and $K_b$, respectively) and allosteric ligands ($K_c$ and $K_d$, respectively), and the equilibrium between the free forms of the states ($R$ and $R^*$) are determined by the constant $K_i$. All equilibriums of the cube can be described using these five constants, which are defined under “Appendix.”

Figure 6 shows how positive ($K_d/K_c = 100$) and negative ($K_d/K_c = 0.01$) allosteric modulators affect the change in observed affinity ($\alpha$) and intrinsic efficacy ($\beta$) of the orthosteric ligand-receptor complex. The values of Log $\alpha$ and $\beta$ are plotted against the log of the ratio of microscopic affinity constants of the orthosteric ligand for active and inactive conformations of the receptor ($K_b/K_a$). In this example, very little of the receptor is in the active state in the absence of agonist ($K_i = 10^{-3}$). The orthosteric ligand can be defined as an inverse agonist ($K_b/K_a < 1$), neutral antagonist ($1 < K_b/K_a < 1/K_i$) or agonist ($K_b/K_a >> 1$), depending upon its selectivity for the ground and active states. Highly efficacious agonists have very large $K_b/K_a$ ratios. Figure 6a shows that a positive allosteric modulator with selectivity for the active state ($K_d/K_c = 100$) exhibits positive cooperativity with an agonist and no cooperativity ($\alpha = 1$) with a neutral antagonist or inverse agonist. A positive modulator would exhibit negative cooperativity with an inverse agonist if the selectivity of the modulator for the active state were sufficient
(\(K_d/K_e > 10^3\)) to overcome the large tendency for the free receptor to exist in the inactive state (\(K_i = 10^3\)). A complementary effect is seen with the negative allosteric modulator; that is, the modulator exhibits negative cooperativity with agonists and no cooperativity (\(\alpha = 1\)) with a neutral antagonist or inverse agonist. In the latter case, there is no cooperativity between an inverse agonist and a negative allosteric modulator because the receptor is already in the inactive state in the absence of ligands. With regard to agonists, the allosteric modulation (\(\alpha\)) of affinity is proportional to the efficacy of the agonist over a range of \(K_d/K_a\) ratios, but reaches a plateau at high ratios. This behavior is well established and has been described previously ((Ehlert et al., 1983; Ehlert, 1986; Hall, 2000)).

Figure 6 also shows the effect of the allosteric modulator on the change in the observed intrinsic efficacy of the orthosteric ligand (\(\beta\)) over a broad range of \(K_d/K_a\) values. The change in \(\beta\) is related to the maximum of the receptor activation function (stimulus) for the orthosteric ligand. The maximum of this function (observed intrinsic efficacy) in the presence of a maximally effective concentration of the modulator divided by that in its absence is defined as \(\beta\) (equation 31). When the orthosteric ligand lacks a sufficiently large \(K_d/K_a\) value to trigger a measurable response, it would be impossible to measure the \(\beta\) value through the analysis of a downstream response. Nonetheless, Figure 6 shows the theoretical \(\beta\) values over the complete range of \(K_d/K_a\) values of the orthosteric ligand. Both positive and negative allosteric modulators cause corresponding changes in observed intrinsic efficacy in addition to their effects on affinity. The product of their combined effect on affinity and efficacy (\(\alpha\beta\)) is constant and equivalent to the ratio of microscopic affinity constants of the allosteric ligand for the ground and active states of the receptor (\(K_f/K_e\)). The basis of this relationship is described under “Appendix” (equation 33). For very weak partial agonists, allosteric modulators primarily affect observed intrinsic efficacy. If the intrinsic efficacy of the orthosteric ligand is high enough so that it is capable of causing nearly complete receptor activation, even in the presence of the modulator (i.e., \(K_b K_d/K_a K_e K_i \geq 10\)), then the allosteric modulation is manifest as a change in observed affinity only.
Figure 7 shows a simulation of the effect of allosteric modulators on the occupancy, stimulus and response functions of an agonist. In Figure 7a, the effects of maximally effective concentrations of positive ($K_f/K_e = 10$) and negative ($K_f/K_e = 0.1$) modulators on the occupancy and stimulus of an agonist ($K_b/K_a = 10^3$) are shown. The positive modulator causes increases in observed affinity and intrinsic efficacy corresponding to factors of (5.5) and (1.8) respectively. The product of these two effects is equivalent to $K_f/K_e (10)$. The negative modulator causes decreases in observed affinity and intrinsic efficacy corresponding to factors of (0.55) and (0.18) respectively, with the product of these two effects being equivalent to $K_f/K_e (0.1)$. Figures 7b and c show the effects of a maximally effective concentrations of the same positive and negative modulators on the response to the agonist in a highly sensitive system exhibiting a large receptor reserve (panel b) and in a less sensitive one (panel c). The concentration-response curves were generated using the operational model as described in the legend to the figure. In the more sensitive system the positive and negative allosteric modulators shift the concentration-response curves 10-fold, without affecting the maximal response. It has been shown previously that this shift is equivalent to the product $\alpha \beta$ (Ehlert, 1988a; Ehlert, 2005) (i.e., 10 for the positive modulator and 0.10 for the negative modulator), which is also equivalent to $K_f/K_e$ (equation 33). In the less sensitive system, the allosteric modulator causes changes in the potency and maximal response of the agonist. It is possible to obtain independent estimates of $\alpha$ and $\beta$ in this condition through analysis of the concentration-response curves as described previously (Ehlert, 2005). For the positive modulator, this analysis yielded estimates of 5.5, 1.8 and 10 for $\alpha$, $\beta$ and their product, respectively. For the negative modulator, the corresponding estimates are 0.18, 0.55 and 0.10.

**Complex two-state model:** We investigated a two-state model incorporating two ground conformations ($R_1$, $R_2$) and two active conformations ($R_1^*$ and $R_2^*$). We refer to this model as the complex two-state model. As an aside, this is the type of model required to explain the phenomenon of ligand directed signaling at the third level of analysis (Ehlert, 2008). Figure 8
shows how the allosteric ternary complex model (Figure 1b) is expanded into the complex two-state model. Each side of the allosteric model is expanded into an elongated rectangle consisting of three squares of equilibria. The four rectangles form the tri-cubic equilibrium shown in Figure 8. The affinity constants of the agonist for the two active conformations (\(R_1^*\) and \(R_2^*\)) are denoted as \(K_b\) and \(K_d\), whereas those for the two inactive conformations (\(R_1\) and \(R_2\)) are \(K_a\) and \(K_c\), respectively. The corresponding affinity constants of the allosteric modulator are denoted as \(K_f\), \(K_h\), \(K_e\), and \(K_g\), respectively. The constants describing the sequential equilibrium between the free forms of the various conformations (\(R_1\), \(R_1^*\), \(R_2\) and \(R_2^*\)) are denoted by \(K_i\), \(K_j\) and \(K_k\). All of the equilibria shown in the model can be described with these 11 constants, which are defined in Figure 8 and under “Appendix” together with the mathematical solution to the model.

The complex two-state model shown in Figure 8 is a simplification of all possible conformational changes. For example, it is entirely possible that ground state \(R_1\) might change directly into active conformation \(R_2^*\) without going through the transition from \(R_1\) to \(R_1^*\) to \(R_2\) to \(R_2^*\). For the purposes of equilibrium, this matter is unimportant because a more complex model incorporating the latter transition would yield the same result as the simpler scheme shown in Figure 8. Thus, the model represents an oversimplification as far as kinetics are concerned, but not with respect to equilibrium behavior. With regard to conformational changes not specified in Figure 8, it is nonetheless possible to denote their equilibrium constants using the constants designated in the figure. For example, the constant governing the equilibrium between \(R_1\) and \(R_2^*\) (i.e., \(R_2^*/R_1\)) is equivalent to \(K_iK_j\).

It is possible to define the second level parameters of the allosteric model (\(K_1\), \(K_2\), \(\alpha\) and \(\beta\)) in terms of the constants of the complex two-state model, and these definitions are shown under “Appendix,” equations 36 – 38, 44. Like the simple two-state model, the complex two-state model predicts changes in both observed affinity and intrinsic efficacy in almost all instances, except for agonists with intrinsic efficacy so great that they are able to cause complete receptor activation in both the presence and absence of the allosteric modulator. Unlike the consequences of the simple two-state model, the effect of the allosteric modulator on the product
\( \alpha \beta \) varies for different orthosteric ligands in the more complex model. The logarithm of the product \( \alpha \beta \) represents a weighted geometric average of the ratios of microscopic affinity constants of the allosteric modulator for the active and inactive states of \( R_1 \) (\( K_f/K_e \)) and \( R_2 \) (\( K_h/K_g \)). Thus, the value of \( \alpha \beta \) is bounded by the interval of \( K_f/K_e \) to \( K_h/K_g \), and it depends upon whether the allosteric modulator shifts the equilibrium in the direction of \( R_1 \) or \( R_2 \), respectively (see Appendix, equations 46 – 47).

Interesting deviations from the simple model occur when the allosteric modulator selects for rare conformations of the receptor in the more complex model. For example, suppose that \( R_1 \) is the most abundant form (99%) of the receptor under basal conditions in the absence of modulator, and that agonist activation occurs mainly through a transition from \( R_1 \) to \( R_1^* \). If the geometric average of the microscopic affinity constants of the allosteric modulator for the \( R_2 \) conformations are much higher affinity than that of the \( R_1 \) conformation, then the allosteric modulator can switch the activation step to an \( R_2 \) to \( R_2^* \) transition. In so doing, the allosteric modulator can change the pharmacological profile of the receptor. That is, in the presence of the modulator, the rank orders of observed affinity and intrinsic efficacy of a group of agonists will change. This result occurs because one would expect that the ratios of \( K_b/K_a \) and \( K_d/K_c \) would vary randomly among a group of orthosteric ligands because the four conformations (\( R_1, R_1^*, R_2 \) and \( R_2^* \)) represent unique structures having unique structure-affinity relationships. In contrast, the simple two-state model predicts that positive allosteric modulators appear to increase the affinity and efficacy of all agonists, whereas negative modulators do the opposite without altering the rank order of observed affinity and intrinsic efficacy.

If it is assumed that the ratio of \( K_i/K_a \) is the same as \( K_d/K_c \) for a given agonist then it is possible for an allosteric modulator to cause affinity-only modulation provided that \( K_e = K_f \neq K_g = K_h \). Figure 9a shows the behavior of the model under these conditions for a negative allosteric modulator. The values of the microscopic affinity constants of the agonist and the allosteric modulator are displayed graphically in Figure 9b. In this simulation, the most abundant form of the receptor under basal conditions is \( R_1 \), and there is very little receptor activation in the absence
of agonist. As shown in Figure 9b, the ratio of affinity constants of the orthosteric agonist for the ground and active conformations of \( R_1 \) \((K_a/K_b = 100)\) is the same as that of \( R_2 \) \((K_c/K_d = 100)\); however, the average overall affinity for the \( R_1 \) pair is greater than that of the \( R_2 \) pair. In contrast the allosteric modulator has equivalent affinities for the ground and active conformations of \( R_1 \) \((K_e/K_f = 1)\) as well as those of \( R_2 \) \((K_g/K_h = 1)\), but its affinities for the \( R_2 \) pair of conformations are greater than those of the \( R_1 \) pair. This opposite selectivity of the agonist and modulator for the \( R_1 \) and \( R_2 \) pairs of conformations results in negative cooperativity as shown in Figure 9a. That is, the allosteric modulator causes a reduction in the observed binding affinity of the agonist. Because of the unique values of the microscopic affinity constants, this reduction in agonist potency occurs with no change in the maximum of the agonist activation curve, illustrating that the modulator has no influence the intrinsic efficacy of the agonist. Affinity-only modulation can also occur with a positive allosteric modulator provided that the orthosteric and allosteric ligands exhibit the same preference for the \( R_1 \) and \( R_2 \) conformations and that the ratios of \( K_a/K_b \) and \( K_c/K_d \) are constant for the agonist and equal to one for the modulator.

This type of affinity-only allosteric modulation occurs because of the unique relationship among the parameters shown in Figure 9b. Other combinations of microscopic constants can yield affinity only modulation. For example, the ratio of agonist affinity constants for the ground and active states of \( R_1 \) and \( R_2 \) could differ provided that the allosteric modulator exhibits the precise degree of selectivity for the pairs of states to offset the difference:

\[
\frac{K_g K_i + K_d K_f K_j K_k}{K_a + K_c K_j} = \frac{K_b K_f K_i + K_a K_g K_i K_j}{K_a K_c + K_e K_g K_j}
\]

It is possible that the activity of the two active states \((R_1^* \text{ and } R_2^*)\) might differ. Affinity-only modulation could still occur in this situation provided that a corresponding difference in the ratio of agonist affinity constants for ground and active states \((K_a/K_b \text{ and } K_c/K_d)\) or those of the allosteric modulator offset the difference in activity of the two states. These solution sets are unique enough to make it unlikely for an orthosteric-allosteric ligand pair to exhibit affinity-only modulation with this model. Moreover, if a pair is found, other agonists will not exhibit affinity...
only modulation unless their ratios of $K_a/K_b$ and $K_c/K_d$ are the same as those of the first pair. This scenario seems highly unlikely because the four conformations of the receptor represent unique structures with unique structure-affinity relationships.

**Tandem two-site, two-state model:** Within the context of a two-state model, it appears that gallamine reduces a component of agonist affinity for both the ground and active states of the receptor to the same extent (Ehlert, 2005). One way to account for this enigma is to consider a model that distributes the total observed affinity of the agonist over two distinct sites – a distal site undergoing agonist-mediated activation and a more proximal site that relays the agonist to the distal activation site. If allosteric modulators affect the affinity of the relay site only, then only a modification in affinity can occur. Such a relay model has been proposed by Jakubik (2000) to account for the two-step kinetics of ligand binding to $M_1$ and $M_2$ muscarinic receptors, and these investigators have suggested that a superficial aspartic acid$^{99}$ in the $M_1$ sequence is part of the relay site. Hulme and coworkers (2003) have suggested that tryptophan$^{157}$ in the $M_1$ sequence may act as a relay site, whereas Matsui and coworkers (1995) have suggested that superficial tryptophan residues 91, 101 and 400 in the $M_1$ sequence may line a cleft that guides orthosteric ligands inwardly to the activation site. These residues are highly conserved across all muscarinic receptor subtypes including the $M_2$. A similar idea has been advanced for the interaction of substrates with acetylcholinesterase (Harel et al., 1993). We have combined the tandem two-site model with a two-state allosteric model to explain the cooperative interactions at the $M_2$ receptor at the level of receptor states (level three analysis). Figure 10 shows how the allosteric model (Figure 1b) can be incorporated into the tandem two-site model (Figure 10b). The tandem two-site model posits that there are two sites on the receptor ($S_1$ and $S_2$) and that the agonist first binds upon $S_1$ and then transfers to $S_2$ where receptor activation occurs. We postulate that there is an allosteric site on $S_1$ that modulates the affinity of orthosteric ligands for the $S_1$ site, but not the $S_2$ site. Thus, the $S_1$ site can exist in two conformations ($S_1$ and $S_1'$), neither of which has an influence on the activation of the receptor. The $S_2$ site can exist in two states,
inactive \( (S_2) \) and active \( (S_2^*) \), and the degree of receptor activation is proportional to the amount of receptors in the active conformation. The microscopic affinity constants of the orthosteric ligand for the two conformations of the relay site \( (S_I \) and \( S_I' \)) are denoted as \( K_a \) and \( K_b \), respectively, and the unimolecular constants describing the transfer of the ligand from the relay site to the two states of the inactive \( (S_2) \) and active \( (S_2^*) \) conformations of the \( S_2 \) site are denoted as \( K_c \) and \( K_d \), respectively. The microscopic affinity constants of the allosteric modulator for the two conformations of the relay site \( (S_I \) and \( S_I' \)) are denoted as \( K_e \) and \( K_f \), respectively. The equilibrium between the two conformations of the free forms of \( S_I \) and \( S_2 \) are independent of each other and denoted by \( K_g \) and \( K_h \), respectively. All of the equilibria shown in the model can be described with these eight constants, which are defined under “Appendix” together with the mathematical solution to the model.

Like the model in Figure 8, the tandem two-site, two-state model is a simplification of all possible conformational changes. For example, it is entirely possible for the \( S_1S_2 \) conformation to change directly into the \( S_1S_2^* \) conformation without going through the transition from \( S_1S_2 \) to \( S_1'S_2 \) to \( S_1'S_2^* \) to \( S_1S_2^* \). For the purposes of equilibrium, this matter is unimportant because a more complex model incorporating the latter transition would yield the same result as the simpler scheme shown in Figure 10.

Figure 11 shows the influence of a positive \( (K_f/K_e > 1) \) and a negative \( (K_f/K_e < 1) \) allosteric modulator on the occupancy and activation curves of an agonist \( (K_d/K_c = 2 \times 10^3) \). In both cases the modulator shifts the occupancy curve without influencing the maximum degree of receptor activation. Consequently, the tandem two-site, two-state model explains affinity-only modulation of agonist responses regardless of the solution set of parameters. Also, since the allosteric regulation occurs at the relay site and not the activation site, the model can account for the well-known observation that the cooperative effects of gallamine are independent of the magnitude of the efficacy of the orthosteric ligand. This property can be appreciated mathematically by examining Figure 10, which shows that the agonist’s propensity to shuttle to the active state \( S_2^* \) \( (K_d) \) relative to inactive state \( S_2 \) \( (K_c) \) is proportional to efficacy, whereas the
cooperativity constant $\alpha$ is unaffected by these constants (see equation 57 under “Appendix”). The model can also explain the reciprocal modulation of the affinity of orthosteric and allosteric ligands. That is, through application of the equations under “Appendix” it can be shown that if gallamine reduces the affinity of $[^3\text{H}]\text{NMS}$, then the tandem two-site two state model predicts that $[^3\text{H}]\text{NMS}$ should reduce the affinity of gallamine to the same extent.

**Relationship of the state models to the allosteric ternary complex model:** It is important to emphasize that the three models investigated are all consistent at the second level of analysis; that is, they are consistent with the allosteric ternary complex model (Figures 1b, 8b and 10b). This model is the level at which allosterism is usually measured experimentally. At this level the models predict that the orthosteric ligand binds according to a simple one-site model exhibiting an observed dissociation constant equivalent to $1/K_1$, with $K_1 = K_{1,R} + K_{1,R}K_{1,A}$ for the tandem two-site model. All three models predict that the effect of the allosteric modulator is to cause a multiplicative change in the observed dissociation constant by the factor $p$ (see Appendix equations 15 – 18, with level two parameters defined in equations 19 - 21 for the simple model and equations 36 – 38 for the complex model; also equations 50 – 53 for the tandem model, with level two parameters defined in equations 54 - 57) (Ehlert, 1988a; Ehlert, 2005):

$$p = \frac{1 + AK_2^2}{1 + A\alpha K_2^2}$$

The simple and complex two-state models also predict that allosteric modulators may cause a multiplicative change in the intrinsic efficacy of the agonist receptor complex equivalent to $q$ (Ehlert, 2005), with $\beta$ defined in equation 31 for the simple two-state model and equation 44 for the complex two-state model:

$$q = \frac{1 + A\alpha\beta K_2^2}{1 + A\alpha K_2^2}$$

In this equation $\beta$ denotes the intrinsic efficacy of the agonist when the allosteric site is occupied, expressed relative to that of the complex when the allosteric site is empty. It may seem that the constants for cooperativity and efficacy ($\alpha$ and $\beta$) are independent parameters that are unique to
the particular orthosteric-allosteric ligand pair. Our analysis shows that the second level parameters $\alpha$ and $\beta$ of the simple two-state models do not vary independently, but are highly correlated. The more complex two-state model also predicts simultaneous changes in both parameters with allosteric modulation although the magnitude of the changes may be uncorrelated. In contrast, the tandem two-site, two-state model for allosterism predicts affinity only modulation provided that the allosteric site is linked only to the relay site.

It is interesting to consider allosterism from different perspectives relating to the definition of affinity and efficacy. Pharmacologists typically define observed affinity and observed intrinsic efficacy as the $EC_{50}$ (observed dissociation constant, $K_{obs}$) and $E_{max}$ (observed intrinsic efficacy, $\varepsilon$) values of the stimulus function (receptor activation) (Furchgott, 1966). Electrophysiologists studying ligand-gated ion channels have defined affinity and efficacy (gating) at a deeper level of analysis (Colquhoun, 1998). Affinity defines how the agonist would bind if it did not induce a conformational change in the receptor, and intrinsic efficacy is defined as the propensity of the agonist to induce a conformational change (i.e., $K_a$ and $K_bK/K_a$, respectively, in the simple two-state model). Tables 4 and 5 list these two different hierarchical definitions of affinity and efficacy for the three different models and describe the influence of a maximally effective concentration of an allosteric modulator on each parameter. At the level of the stimulus (second level, Table 4), both two state-models predict that an allosteric modulator simultaneously influences both the observed affinity and observed intrinsic efficacy of the agonist, whereas the tandem two-site, two-state model predicts affinity-only modulation. At the level of receptor states (third level), the definition of intrinsic efficacy is such that simple two-state model predicts efficacy-only modulation; the tandem two-site, two-state model predicts affinity-only modulation; and the complex two-state model predicts simultaneous changes in affinity and efficacy. With the latter model, widespread affinity only modulation is extremely remote because the four distinct conformations exhibit four unique structure-affinity relationships (random relationship between $K_a$, $K_b$, $K_e$ and $K_f$ for a sample of orthosteric ligands).
Analysis of allosterism at G protein linked receptors and ligand-gated ion channels using two-state models: The allosteric ternary complex model has been extended previously to account for ground and active conformations of the receptor. In the model of Hall (2000), two-states of the receptor are proposed, but the states are allowed to assume any conformation, which leads to the introduction of cooperativity constants that are undefined in terms of microscopic constants for receptor conformations. This convention has led to the assumption that the cooperativity constants for affinity ($\alpha$) and efficacy ($\beta$) modulation ($\gamma$ and $\delta$, respectively in the model of Hall (2000)) are independent parameters of two-state allosteric models. As described above, however, these parameters are not independent in two-state models, and affinity-only modulation as defined in Table 5 is an unlikely outcome of such a model. As illustrated with the behavior of gallamine, the models described in this report enable one to address the origin of cooperativity in two-state models and to assess what mechanisms can account for the measured cooperativity.

For example, one phenomenon that is thought to be a consequence of the extended allosteric ternary complex model is the ability of two neutral ligands to interact allosterically to produce a response even though each ligand lacks selectivity for the active state (Hall, 2000). According to the complex two-state allosteric model, this phenomenon could occur if there were two pairs ($R_1$ and $R_2$) of ground and active states in equal abundance ($K_j = 1$) and the orthosteric ligand acted as an inverse agonist at $R_1$ ($K_b < K_a$) and an agonist at $R_2$ ($K_d > K_c$). The net effect is that inverse agonism at $R_1$ is opposed by agonism at $R_2$ resulting in no net receptor activation. If the allosteric ligand behaved like that shown in Figure 9 and selected for the $R_2$ pair without activating it ($K_g = K_h > K_e = K_f$), then the pair of ligands could produce a response together, although either ligand by itself would have no effect on receptor activation. The same phenomenon could occur if the roles of the ligands were reversed. This exotic explanation seems unlikely, however. The simple two-state model readily accounts for the phenomenon of coagonism in terms of positive cooperativity between two interacting ligands – each exhibiting selectivity for the active state ($K_b/K_a > 1$ and $K_d/K_c > 1$) although the amount of selectivity is insufficient for either one to elicit a response by itself ($K_b/K_a << 1/K_i$ and $K_d/K_c << 1/K_i$). An
analogous form of homotropic cooperativity is commonplace at oligomeric, ligand-gated ion channels. Often, the binding of more than one agonist molecule is required for channel opening (e.g., nicotinic acetylcholine receptor).

It has also been suggested that the extended allosteric ternary complex model can account for the phenomenon whereby a modulator increases the affinity ($\alpha > 1$) and reduces the efficacy ($\beta < 1$) of an agonist simply by changing the amount of active receptor complex and its observed affinity (Hall, 2000). According to the complex two-state model, this phenomenon could occur if there were two pairs of agonist conformations ($R_1$ and $R_2$), and the agonist activated the more abundant pair ($R_1$, $K_b \gg K_a$), but behaved as an inverse agonist ($R_2$, $K_d << K_c$) with much higher affinity ($K_d > K_a$) at the less abundant pair of conformations ($K_j << 1$). If the modulator behaved like that shown in Figure 9 and selected for the less abundant pair of conformations ($K_g = K_h > K_e = K_f$), then it would cause an increase in affinity and decrease in efficacy of the agonist-receptor complex. Once again, however, such a complicated mechanism seems unlikely.

A much simpler mechanism is the phenomenon of uncompetitive inhibition, whereby the allosteric modulator exhibits selectivity for the active state, yet inhibits activity when bound to it. For a ligand-gated ion channel, the mechanism of this inhibition could be an open channel block. For a G protein coupled receptor, the inhibitor could sterically interfere with the receptor-G protein interaction.

It has also been argued that the extended ternary complex model shows that the allosteric parameters are system-dependent whenever the orthosteric ligand exhibits selectivity for ground or active conformations of the receptor (Hall, 2000). For the simple two-state model, however, we show that the product $\alpha \beta$ is equivalent to $K_f/K_e$ provided that $K_i << 1$ for negative modulators and that $K_f/K_e << 1/K_i$ for positive modulators. Since it is always possible to estimate the product of $\alpha \beta$ in functional studies (Ehlert, 1988a; Ehlert, 2005), then it should be possible to measure the selectivity of the allosteric modulator for ground and active states of the receptor ($K_f/K_e$) for any system conforming to the simple two-state model. The latter ratio is solely a property of the allosteric ligand-receptor complex.
DISCUSSION

Two-state models inevitably predict that allosteric modulators affect the observed efficacy of the agonist. This report as well as those of others have generated a small list of agonists (acetylcholine, S-aceclidine, arecoline, BM5, carbachol and oxotremorine-M) whose efficacy is unaffected by gallamine. These results would appear to rule out two-state models as the sole explanation for the allosteric effect of gallamine.

An issue requiring careful consideration in a two-state model relates to the maximal degree of receptor activation. Highly efficacious agonists that cause complete receptor activation - even in the presence of a negative modulator - would only exhibit an allosteric modulation in affinity. When M2 muscarinic receptor mediated inhibition of adenylyl cyclase activity was measured in homogenates of the myocardium in the presence of 0.1 mM GTP, the relative efficacies values of a group of agonists all varied, with the values for oxotremorine-M, carbachol, S-aceclidine, and BM5 being 1.6, 1, .37 and 0.087, respectively (Ehlert, 1985). The lack of a clear plateau value for the efficacy of the most efficacious agonists suggests that none of the agonists cause maximal receptor activation. This result is not surprising because the assays were carried out in the presence of GTP (0.1 mM), which greatly inhibits the proportion of the agonist-receptor complex in the active state (Ehlert, 2000). It is possible that the intracellular concentration of GTP is comparatively lower in CHO cells, which would enable agonists with lower efficacy to generate more of the active receptor conformation. This postulate is consistent with the moderate increase in the observed affinity of oxotremorine-M observed in this study ($pK_{obs} = 6.03$) compared to that observed in myocardial homogenates in the presence of GTP (0.1 mM) ($pK_{obs} = 5.12$) (Ehlert, 1987). The moderate increase in affinity indicates that the corresponding increase in the proportion of occupied receptor in the active conformation is also moderate, which suggests that the agonist $K_d/K_a$ ratios are not much greater than that required for maximal receptor activation in the CHO cell. If the agonists were near the threshold for complete receptor activation, then one would expect that gallamine would still cause a reduction in
receptor activation at high concentrations (10 – 100 µM) if the two-state model were tenable. The result would be a greater gallamine-induced reduction in agonist potency for inhibiting cAMP accumulation as compared to that for observed binding affinity (see Figure and (Ehlert, 2005)) or an accompanying decline in $E_{max}$. This behavior was not observed, however, and the shift in the agonist-concentration-response curve was equivalent to the measured change in binding affinity. Also high concentrations of gallamine (1 – 0.1 mM) were without effect on the $E_{max}$ of the partial agonist BM5 (Ehlert, 1988b) or on the $E_{max}$ of oxotremorine-M after a reduction in its $E_{max}$ by partial receptor inactivation (Figure 5).

The simple two-state model predicts a correlation between the cooperativity constant ($\alpha$) for a given allosteric-orthosteric ligand pair and the intrinsic efficacy of the orthosteric ligand. In other words, the model predicts that the absolute value of the Log $\alpha$ value would be greatest for efficacious agonists, intermediate for agonists with low efficacy and zero for antagonists. Interestingly, it also predicts that the product of the cooperativity constants for observed affinity ($\alpha$) and intrinsic efficacy ($\beta$) is constant and equivalent to the ratio of microscopic affinity constants of the modulator for active and inactive states of the receptor ($K_f/K_e$). Various GABA$_A$ receptor subtypes exhibit this behavior with regard to the allosteric interactions between GABA agonists and benzodiazepine, barbiturates and other allosteric modulators (Ehlert et al., 1983; Levitan et al., 1988; Sigel and Baur, 1988). In contrast, gallamine exhibits substantial negative cooperativity with both agonists and antagonists, which is inconsistent with the simple two-state model (Stockton et al., 1983). The complex two-state model can explain this behavior; however, it cannot account for widespread modulation in affinity only. In contrast, the tandem two-site, two-state allosteric model can account for the behavior of gallamine.

At the present time, we cannot rule out small allosteric changes in the intrinsic efficacy of the agonist receptor complex by gallamine. The data of Jakubik et al. (1996) showing that occupancy of the allosteric site on M$_2$ receptors by gallamine actually elicits small agonistic effects (i.e., inhibition in cAMP accumulation) suggests that a minor effect on efficacy may occur. Within the context of the tandem two-site model, the latter observation may imply that
conformational changes in the relay site and activation site are not completely independent of each other.

There are several examples of other allosteric muscarinic ligands that behave differently from gallamine, indicating that not all allosteric interactions at the M₂ muscarinic receptor can be explained by the tandem two-site, two-state allosteric model as described here. For example, alcuronium has been shown to modulate the intrinsic efficacy of pilocarpine (Zahn et al., 2002) and the allosteric effects of strichnyne on acetylcholine-stimulated \[^{35}\text{S}\]GTP\(\gamma\)S binding show a discrepancy between the allosteric modulation of observed binding affinity and functional potency indicating allosteric changes in agonist efficacy (Lazareno and Birdsall, 1995). It is generally assumed that alcuronium acts at the same allosteric site as gallamine, and it is conceivable that occupancy of the site by alcuronium alters the unimolecular constants \((K_c, K_d)\) of the activation site such that efficacy modulation occurs. Also, the compound AC-42 (4-n-Butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine) has been shown to activate the M₂ muscarinic receptor, and it appears to do so through binding to an allosteric site located on the extracellular loops of the receptor (May et al., 2007). The muscarinic agonist McN-A-343 has also been suggested to act allosterically (Birdsall et al., 1983; May et al., 2007). If the mechanism were allosteric, then it would exhibit a high degree of negative cooperativity with both NMS and carbachol. In the case of carbachol, the minimum estimate of the absolute value of the logarithm of its \(\alpha\) value with carbachol would be approximately 3.0 in order to explain the data obtained by Christopolous and Michelson (1997). Since McN-A-343 activates the M₂ receptor it must discriminate between active and inactive states. An allosteric mechanism would require these conformations to be different from those utilized by carbachol otherwise the two agonists would exhibit positive cooperativity.

Models of the M₂ receptor place the location of the allosteric site at the level of the outer membrane surface, whereas the orthosteric site is thought to be deeper within the transmembrane domains (Trankle et al., 2005). This topography explains why gallamine and other allosteric agents greatly slow the binding kinetics of orthosteric ligands, because the model predicts that
the allosteric ligand may trap the orthosteric ligand when both types of ligands are occupying their respective sites. Jakubik et al. (2000) have suggested that the putative relay site and activation site are in such close proximity that occupancy of both sites by two molecules of NMS is sterically hindered. If the putative relay site facilitates the inward transfer of orthosteric ligands to the activation site, then it would seem that the relay site might be between the activation and allosteric sites. Perhaps the conformational states of the putative relay site function normally in the absence of allosteric modulators to facilitate the inward transfer of agonist to the activation site.

It has been shown that the second extracellular loop (E2) of the M2 muscarinic receptor has modest role in the binding of gallamine, but a much greater role in the binding of other modulators (Leppik et al., 1994; Gnagey et al., 1999; Voigtlander et al., 2003). Cysteine mutagenesis of residues valine\textsuperscript{171} and asparagine\textsuperscript{419} caused a marked reduction in the affinity of acetylcholine and only a moderate reduction in that of gallamine (Avlani et al., 2007). These effects were reversed by treatment with dithiothreitol suggesting that the changes in affinity are caused by bending the E2 loop over the binding pocket of the receptor through a disulfide bond linkage. Since the E2 loop seems far from what is thought to be the acetylcholine binding pocket, the results suggest that a reduction in agonist affinity can be caused by reducing access to the binding pocket by a greater net decrement relative to egress (outward rectification). This “capping” mechanism is reminiscent of the slowing in the kinetics of [\textsuperscript{3}H]NMS binding caused by gallamine (Stockton et al., 1983). It is conceivable that the postulated allosteric effect of gallamine on the putative relay site may cause a relative outward rectification of access to the agonist binding pocket similar to that caused by modification of the E2 loop.

Our models are straightforward extensions of the original ideas put forth by Monod, Wyman and Changeux (Monod et al., 1965) and Koshland and coworkers (Koshland et al., 1966) and have widespread application to the analysis of allostery at other receptors. A better understanding of the origin of cooperativity and the interdependence of allosteric changes in affinity and efficacy should help in the identification of accurate models for allostery at ligand-
gated ion channels and G protein coupled receptors.
REFERENCES


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol.* 22:3099-3108.


FOOTNOTES

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Legends for Figures

Figure 1: Hierarchical levels of analysis and the simple two-state model for allostereism. 

a: On the surface, the binding of drug D to the receptor is consistent with a simple one-site model characterized by an observed dissociation constant ($K_{obs}$). 
b: At the second level of analysis, the bound drug can be divided into two types of complexes, a binary drug-receptor complex ($DR$) and a ternary drug-receptor-modulator complex ($DRA$), exhibiting microscopic affinity constants of $K_1$ and $\alpha K_1$, respectively. The concentration of D required for half-maximal formation of each complex is equivalent to $K_{obs}$. 
c: At the third level of analysis, each type of receptor complex can be further divided into two states, ground ($R$) and active ($R^*$), each exhibiting a unique, unvarying microscopic affinity constant. The concentration of D required for half-maximal formation of the two states of each of the two types of drug receptor complexes is equivalent to $K_{obs}$.

Figure 2: Expansion of the allosteric ternary complex model into various two-state models. 

a, Simple two-state model: The receptor complex exists in two states, inactive and active. In the absence of ligands, the inactive state of the receptor predominates. Agonists and positive allosteric modulators bind to their respective orthosteric and allosteric sites and exhibit selectivity for the active state, whereas negative modulators exhibit selectivity for the inactive state. 
b, Complex two-state model: The receptor complex exists in two states, inactive and active, each with two different conformations. In the absence of ligands, the two conformations of the inactive state of the receptor predominate. Agonists and positive allosteric modulators bind to their respective orthosteric and allosteric sites and exhibit selectivity for the active conformations, whereas negative modulators exhibit selectivity for the inactive conformations. 
c, Tandem two-site, two-state model: The receptor complex has a superficially located relay site ($S_1$) that directs the agonist to the activation site ($S_2$). The relay site exists in two conformations.
that are linked to an allosteric binding site. Positive allosteric modulators select for a conformation that exhibits high affinity for the orthosteric ligand and negative allosteric modulators select for a conformation that exhibits low affinity for the orthosteric ligand. The activation site exists in two states, inactive and active, which undergo conformational changes independently of the relay site. In the absence of an orthosteric ligand, the inactive state predominates. Agonists exhibit selectivity for the active state.

**Figure 3:** *The Influence of gallamine on the specific binding of $[^3\text{H}]$NMS to the human M$_2$ muscarinic receptor stably expressed in CHO cells.*

- **a:** The specific binding of $[^3\text{H}]$NMS was measured at various concentrations of radioligand and in the absence and presence of gallamine (10 µM). The data represent the mean binding values ± SEM of four experiments, each done in triplicate.
- **b:** The specific binding of $[^3\text{H}]$NMS was measured at a fixed concentration (0.5 nM) in the presence of various concentrations of gallamine. The data represent the mean binding values ± SEM of three experiments, each done in triplicate.

**Figure 4:** *The influence of gallamine on agonist/$[^3\text{H}]$NMS binding competitive binding curves.*

The specific binding of $[^3\text{H}]$NMS was measured at a fixed concentration (0.9 nM) in the absence and presence of various concentrations of acetylcholine (a), carbachol (b), oxotremorine-M (c), S-aceclidine (d) and arecoline (e). The experiments were repeated in the presence of the indicated concentrations of gallamine. The competition curves have been scaled to the maximum specific binding measured in the absence of agonist. The data represent the mean binding values ± SEM of five (a – c) and three (d, e) experiments, each done in triplicate.

**Figure 5:** *The influence of gallamine on agonist-mediated inhibition of forskolin stimulated cAMP accumulation in CHO cells stably expressing the human M$_2$ muscarinic receptor.* Cyclic AMP accumulation was measured in the presence of a fixed concentration of forskolin (10 µM) in the absence and presence of various concentrations of acetylcholine (a), carbachol (b), oxotremorine-M (c), S-aceclidine (d), arecoline (e) and oxotremorine-M, before and after
treatment with 4-DAMP mustard (f). The experiments were repeated in the presence of the indicated concentrations of gallamine. The data represent the mean values ± SEM of four experiments, each done in triplicate.

**Figure 6:** *Simulation of the simple two-state model for allosterism.* a, The logarithm of the cooperativity constants describing the change in observed affinity (α) and intrinsic efficacy (β) of the agonist-receptor-positive allosteric modulator complex is plotted against the logarithm of the ratio of microscopic affinity constants of the orthosteric ligand for the active (K_b) and inactive (K_a) states of the receptor. Also shown is the product of the cooperativity constants (αβ). For this simulation, very little receptor (0.1%) is in the active state in the absence of orthosteric ligand (K_i = 10^{-3}). The ratio of microscopic affinity constants of the allosteric ligand for the active (K_f) and inactive (K_e) states of the receptor are indicated in the figure. b, The same as a except that the corresponding values for a negative allosteric modulator are shown. The α and β values were estimated using equations 21 and 31, respectively.

**Figure 7:** *Simulation of the influence of maximally effective concentrations of positive and negative allosteric modulators on the occupancy, stimulus and response of an agonist.* a, The effects of positive (1 mM) and negative (1 mM) modulators on the occupancy (open symbols) and stimulus (closed symbols) curves for an agonist are shown. For this simulation, very little receptor (0.1%) is in the active state in the absence of agonist (K_i = 10^{-3}). The microscopic affinity constants of the agonist for the ground and active conformations of the receptor are K_a = 10^4 and K_b = 10^7. The microscopic affinity constants of the positive allosteric modulator for the ground and active states are K_e = 10^8 and K_f = 10^9, and those of the negative allosteric modulator are K_e = 10^9 and K_f = 10^8. These microscopic constants yield the following second level parameters for the agonist and the positive and negative allosteric modulators, K_1 = 2.0 \times 10^4, K_2 = 1.0 \times 10^8 and K_2 = 1.0 \times 10^9, respectively. The cooperativity (α) between the binding of agonist and positive and negative allosteric modulators are 5.5 and 0.55, respectively.
observed efficacies of the agonist in the presence of the positive and negative modulators expressed relative to control are 1.8 and 0.18, respectively. The observed dissociation constant of the agonist in the absence and presence of the positive (1 mM) and negative (1 mM) allosteric modulators are 5.0, 1.5 and 8.5 µM, respectively. The occupancy and stimulus functions for the agonist were calculated using equations 14 and 25, respectively. b, The effects of positive (1 mM) and negative (1 mM) modulators on a highly sensitive response to an agonist. The concentration response curves were generated using the operational model (response = \(S/(S + 0.001)\)) (Black and Leff, 1983), in which \(S\) denotes the stimulus plotted in panel a. c, The effects of positive (1 mM) and negative (1 mM) modulators on a less sensitive response to an agonist. The simulation was done as described in b, except that the response was generated from a less sensitive operational model. The concentration response curves were generated using the operational model (response = \(S/(S + 0.1)\)).

**Figure 8:** Hierarchical levels of analysis and the complex two-state model for allosteryism. a: On the surface, the binding of drug \(D\) to the receptor is consistent with a simple one-site model characterized by an observed dissociation constant (\(K_{obs}\)). b: At the second level of analysis, the bound drug can be divided into two types of complexes, a binary drug-receptor complex (\(DR\)) and a ternary drug-receptor-modulator complex (\(DRA\)), exhibiting microscopic affinity constants of \(K_1\) and \(\alpha K_1\), respectively. The concentration of \(D\) required for half-maximal formation of each complex is equivalent to \(K_{obs}\). c: At the third level of analysis, each type of receptor complex can be further divided into four states, two ground (\(R_1\) and \(R_2\)) and two active (\(R_1^*\) and \(R_2^*\)), each exhibiting a unique, unvarying microscopic affinity constant. The concentration of \(D\) required for half-maximal formation of the four states of each of the two types of drug receptor complexes is equivalent to \(K_{obs}\).

**Figure 9:** A unique set of parameter values, albeit unlikely, enables the complex two-state model to account for a selective allosteric modulation in agonist affinity without affecting
intrinsic efficacy. a: The effects of a negative modulator on the occupancy (open symbols) and stimulus (closed symbols) curves for an agonist are shown. For this simulation, the relative abundances of the various receptor states (R₁, R₁*, R₂ and R₂*) in the absence of agonist are 99.8, 0.1, 0.1 and 0.0001%, respectively (Kᵢ = Kᵢ = Kᵢ = 10⁻³). The microscopic affinity constants of the agonist for the two ground (Kₐ and Kₖ) and two active (Kₖ and Kₖ) conformations of the receptor as well as those of the negative modulator (Kₑ, Kₑ, Kₑ and Kₑ, respectively) are illustrated in panel b. These microscopic constants yield the following second level parameters for the agonist and negative allosteric modulator, Kᵢ = 2.0 x 10⁵, K₂ = 1.1 x 10⁶ and α = 0.10. The observed dissociation constant of the agonist in the absence and presence of the negative allosteric modulator (1 mM) are 5.0 and 50 µM, respectively.

**Figure 10:** Hierarchical levels of analysis and the tandem two-site, two-state model for allosterism. a: The tandem two-site model posits that the drug D first binds upon the receptor at relay site S₁ and then shuttles to the activation site S₂. On the surface, the binding of drug D to this circuit of two sites is consistent with a simple one-site model characterized by an observed dissociation constant (K(obs)) equivalent to 1/(K₁R + K₁R K₁A). b: At the second level of analysis, bound drug can be divided into two main types of complexes, a binary drug-receptor complex and a ternary drug-receptor-modulator complex. Each of these can be further divided, depending upon whether the drug is occupying the relay site (DS₁S₂ and DS₁AS₂) or the activation site (S₁DS₂ and S₁ADS₂). The concentration of D required for half-maximal formation of each complex is equivalent to K(obs). c: At the third level of analysis, each type of receptor complex can be further divided based on the state of the relay site (S₁ and S₁') and the activation site (S₂ and S₂*), each exhibiting a unique, unvarying microscopic affinity constants. The concentration of D required for half-maximal formation of the various combinations of states of the various types of drug-receptor complexes is equivalent to K(obs).

**Figure 11:** The tandem two-site, two-state model readily accounts for a selective modulation in agonist affinity by negative and positive allosteric modulators. The effects of positive and
negative modulators on the occupancy (open symbols) and stimulus (closed symbols) curves for an agonist are shown. For this simulation, very little (0.1%) of the activation site is in the active state \( (S_2^*) \) in the absence of agonist \( (K_h = 10^{-3}) \) and very little (1%) of the relay site is in the \( S_1' \) conformation in the absence of ligands \( (K_g = 10^{-2}) \). The microscopic affinity constants of the agonist for the \( S_1 \) and \( S_1' \) conformations of the relay site are \( K_a = 10^3 \) and \( K_b = 10^7 \), respectively. The corresponding values for the positive modulator are \( K_c = 10^5 \) and \( K_f = 10^8 \) and those of the negative modulator are \( K_e = 10^8 \) and \( K_f = 10^5 \). The unimolecular constants describing the transfer of the orthosteric ligand to the ground and active conformations of the activation site are \( K_c = 10 \) and \( K_d = 2 \times 10^4 \), respectively. These microscopic constants yield the following second level parameters for the agonist and the positive and negative allosteric modulators, \( K_{1-R} = 10^5 \), \( K_{1-A} = 30 \) and \( K_2 = 9.9 \times 10^7 \), respectively. The cooperativity \( (\alpha) \) between the binding of agonist and positive and negative allosteric modulators are 91 and 0.011, respectively. The observed dissociation constant of the agonist in the absence and presence of the positive (1 mM) and negative (1 mM) allosteric modulators are 0.32, 0.0035 and 29 µM, respectively. The occupancy and stimulus functions were calculated using equations 50 and 63, respectively.
Table 1: Binding parameters of muscarinic ligands at the human M₂ muscarinic receptor

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Orthosteric site Log K₁</th>
<th>Allosteric site Log K₂</th>
<th>Cooperativity with gallamine (α)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[³H]NMS</td>
<td>9.84 ± 0.038</td>
<td>-</td>
<td>0.065 (-1.19 ± 0.050)</td>
</tr>
<tr>
<td>Gallamine</td>
<td>-</td>
<td>6.25 ± 0.064</td>
<td>-</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>5.93 ± 0.073</td>
<td>-</td>
<td>0.013 (-1.88 ± 0.10)</td>
</tr>
<tr>
<td>S-Aceclidine</td>
<td>4.75 ± 0.029</td>
<td>-</td>
<td>0.048 (-1.32 ± 0.13)</td>
</tr>
<tr>
<td>Arecoline</td>
<td>4.66 ± 0.014</td>
<td>-</td>
<td>0.0058 (-2.23 ± 0.16)</td>
</tr>
<tr>
<td>Carbachol</td>
<td>5.12 ± 0.062</td>
<td>-</td>
<td>0.0090 (-2.05 ± 0.12)</td>
</tr>
<tr>
<td>Oxotremorine-M</td>
<td>5.67 ± 0.077</td>
<td>-</td>
<td>0.0082 (-2.09 ± 0.12)</td>
</tr>
</tbody>
</table>

The data are calculated from the experiments shown in Figures 3 and 4. The number of experiments is given in the appropriate figure legends. The data represent mean values ± SEM. The SEM values of the agonist parameters were calculated assuming constant values for K₂, the K₁ of [³H]NMS and α value for the gallamine-[³H]NMS interaction.

The values in parentheses beneath each estimate represent the Log mean ± SEM.
Table 2: Competitive inhibition of the binding of \[^{3}H\]NMS to human M\(_2\) muscarinic receptors by muscarinic agonists in the presence and absence of gallamine (10 \(\mu\)M)\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>(pIC_{50})^b</th>
<th>Hill slope</th>
<th>(pIC_{50})^b</th>
<th>Hill slope</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>5.07 ± 0.073</td>
<td>0.99 ± 0.032</td>
<td>4.52 ± 0.071</td>
<td>1.09 ± 0.026</td>
</tr>
<tr>
<td>S-Aceclidine</td>
<td>3.87 ± 0.029</td>
<td>0.91 ± 0.054</td>
<td>3.51 ± 0.050</td>
<td>0.93 ± 0.089</td>
</tr>
<tr>
<td>Arecoline</td>
<td>3.71 ± 0.014</td>
<td>0.95 ± 0.050</td>
<td>3.33 ± 0.028</td>
<td>1.1 ± 0.012</td>
</tr>
<tr>
<td>Carbachol</td>
<td>4.27 ± 0.062</td>
<td>0.98 ± 0.039</td>
<td>3.69 ± 0.071</td>
<td>1.00 ± 0.046</td>
</tr>
<tr>
<td>Oxotremorine-M</td>
<td>4.82 ± 0.077</td>
<td>0.90 ± 0.054</td>
<td>4.23 ± 0.062</td>
<td>0.96 ± 0.032</td>
</tr>
</tbody>
</table>

\(^a\) The data are calculated from the experiments shown in Figure 4. The number of experiments is given in the legend to Figure 4. The data represent mean values ± SEM.

\(^b\) Denotes the negative logarithm of the molar \(IC_{50}\) value.
Table 3: Summary of the effects of gallamine on the function (inhibition of cAMP) and binding affinity of muscarinic agonists

<table>
<thead>
<tr>
<th></th>
<th>Maximal Inhibition of cAMP (%)</th>
<th>pEC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Observed Log Shift</th>
<th>Predicted Log Shift binding data</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acetylcholine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>61 ± 2.8</td>
<td>7.30 ± 0.067</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gallamine 10 µM</td>
<td>66 ± 3.9</td>
<td>6.16 ± 0.049</td>
<td>1.22 ± 0.087</td>
<td>1.19 ± 0.025</td>
</tr>
<tr>
<td>S-Aceclidine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>60 ± 1.3</td>
<td>5.99 ± 0.059</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gallamine 10 µM</td>
<td>60 ± 3.5</td>
<td>4.89 ± 0.18</td>
<td>1.12 ± 0.11</td>
<td>1.01 ± 0.066</td>
</tr>
<tr>
<td>Arecoline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>46 ± 4.1</td>
<td>6.09 ± 0.084</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gallamine 3 µM</td>
<td>47 ± 3.5</td>
<td>5.55 ± 0.12</td>
<td>0.59 ± 0.17</td>
<td>0.79 ± 0.092</td>
</tr>
<tr>
<td></td>
<td>46 ± 3.3</td>
<td>4.82 ± 0.15</td>
<td>1.28 ± 0.079</td>
<td>1.22 ± 0.041</td>
</tr>
<tr>
<td></td>
<td>42 ± 4.9</td>
<td>4.07 ± 0.21</td>
<td>1.99 ± 0.18</td>
<td>2.02 ± 0.069</td>
</tr>
<tr>
<td>Carbachol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>65 ± 2.3</td>
<td>6.40 ± 0.069</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gallamine 10 µM</td>
<td>64 ± 1.7</td>
<td>5.32 ± 0.018</td>
<td>1.08 ± 0.062</td>
<td>1.21 ± 0.029</td>
</tr>
<tr>
<td>Oxotremorine-M</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>67 ± 4.4</td>
<td>7.06 ± 0.087</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gallamine 10 µM</td>
<td>72 ± 1.4</td>
<td>5.92 ± 0.043</td>
<td>1.14 ± 0.059</td>
<td>1.22 ± 0.025</td>
</tr>
<tr>
<td></td>
<td>100 µM</td>
<td></td>
<td>1.94 ± 0.094</td>
<td>1.86</td>
</tr>
</tbody>
</table>

The predicted Log Shift has been calculated from the binding data shown in Figures 3, 4 and 5 and Table 1. The SEM values for the theoretical Log shift in binding affinity were estimated assuming constant values for $K_2$, the $K_I$ of [³H]NMS and $\alpha$ value for the gallamine-[³H]NMS interaction.
Table 4: Relationship between the microscopic constants of the allosteric ternary complex model and the definitions of affinity and efficacy at the second level of analysis. The table shows the maximal effect of the modulator on affinity and efficacy.

<table>
<thead>
<tr>
<th>Model</th>
<th>Second level analysis (stimulus)</th>
<th>Observed affinity $^a$</th>
<th>Observed intrinsic efficacy $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple two-state control plus modulator</td>
<td></td>
<td>$\frac{1 + K_j}{K_a + K_b K_i}$</td>
<td>$\frac{1}{1 + \frac{K_a}{K_b K_i}}$</td>
</tr>
<tr>
<td>Complex two-state control plus modulator</td>
<td>$\frac{K_e + K_J K_i}{K_a K_e + K_b K_f K_i}$</td>
<td>$\frac{1}{1 + \frac{K_b K_e}{K_b K_f K_i}}$</td>
<td>$\frac{1}{1 + \frac{K_a + K_J}{K_b K_f K_i}}$</td>
</tr>
<tr>
<td>Tandem two-site, two-state control plus modulator</td>
<td>$\frac{(1 + K_h)(1 + K_g)}{(1 + K_e + K_h + K_a K_h)(K_a + K_b K_g)}$</td>
<td>$\frac{1}{1 + \frac{K_e}{K_d K_h}}$</td>
<td>$\frac{1}{1 + \frac{K_e}{K_d K_h}}$</td>
</tr>
</tbody>
</table>

$^a$ Denotes $K_{obs}$ in units of molarity.

$^b$ Denotes the maximum of the stimulus function.
Table 5: Relationship between the microscopic constants of the allosteric ternary complex model and the definitions of affinity and efficacy at the third level of analysis. The table shows the maximal effect of the modulator on affinity and efficacy.

<table>
<thead>
<tr>
<th>Model</th>
<th>Third level analysis</th>
<th>Affinity $^a$</th>
<th>Intrinsic Efficacy $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple two-state control plus modulator</td>
<td></td>
<td>$1/K_a$</td>
<td>$K_b \times K_i$</td>
</tr>
<tr>
<td>Complex two-state control plus modulator</td>
<td></td>
<td>$\frac{1 + K_j}{K_a + K_j K_c}$</td>
<td>$\frac{K_b K_f K_i + K_d K_f K_k}{K_a + K_c K_j}$</td>
</tr>
<tr>
<td>Tandem two-site, two-state control plus modulator</td>
<td></td>
<td>$\frac{1 + K_g}{K_a K_c + K_b K_c K_g}$</td>
<td>$\frac{K_d \times K_b}{K_c}$</td>
</tr>
</tbody>
</table>

$^a$ Affinity is expressed in units of molarity, and it represents the observed affinity of the agonist if did not activate the receptor (e.g., $K_{obs}$ when $K_i = 0$ for the simple two-state model).

$^b$ Intrinsic efficacy is a measure of the agonist’s ability to activate the receptor. Agonist activation of the receptor is defined by equations 28, 41 and 63 for the three models. At high agonist concentrations these equations are of the form $1/(1 + \text{efficacy})$, with efficacy being equivalent to intrinsic efficacy in the Table above.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6

(a) Positive modulator
$K_f/K_e = 100$

(b) Negative modulator
$K_f/K_e = 0.01$

$\alpha \beta = K_f/K_e$

Log Parameter vs. Log $K_b/K_a$
Figure 7
Figure 8
**Figure 9**

(a) Occupancy or stimulus (% maximum) vs. Log [Agonist].

- **Occupancy**:
  - ○ Control
  - △ Negative modulator

- **Stimulus**:
  - ● Control
  - ▲ Negative modulator

(b) Negative modulator

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<tr>
<th>Agonist</th>
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<th>$K_f$</th>
<th>$K_g$</th>
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Figure 10

a  \[ D + S_1 S_2 \xrightarrow{K_{1R}} DS_1 S_2 \xrightarrow{K_{1A}} S_1 DS_2 \]

b  \[ D + S_1 S_2 + A \xrightarrow{K_{1R}} DS_1 S_2 + A \xrightarrow{K_{1A}} S_1 DS_2 + A \]

\[ D + S_1 AS_2 \xrightarrow{\alpha K_2} DS_1 AS_2 \]

\[ \alpha K_2 \]

\[ D + S_1 AS_2 \xrightarrow{K_2} DS_1 AS_2 \]

\[ \alpha K_1 R \]

\[ DS_1 AS_2 \xrightarrow{K_1 A} S_1 ADS_2 \]

\[ S_1 ADS_2 \]

\[ D + S_1 S_2^* + A \xrightarrow{K_a} DS_1 S_2^* + A \xrightarrow{K_d} S_1 DS_2^* + A \]

\[ D + S_1 iAS_2^* \xrightarrow{K_b} DS_1 iAS_2^* \xrightarrow{K_c} S_1 iADS_2^* \]

\[ D + S_1 iAS_2 \xrightarrow{K_b} DS_1 iAS_2 \xrightarrow{K_c} S_1 iADS_2 \]

\[ K_{d_h} \]

\[ K_{d_h} \]

\[ D + S_1 S_2 + A \xrightarrow{K_b} DS_1 S_2 + A \xrightarrow{K_c} S_1 DS_2 + A \]

\[ D + S_1 AS_2 \xrightarrow{K_a} DS_1 AS_2 \xrightarrow{K_c} S_1 ADS_2 \]

\[ D + S_1 AS_2 \xrightarrow{K_a} DS_1 AS_2 \xrightarrow{K_c} S_1 ADS_2 \]

\[ D + S_1 S_2 \xrightarrow{K_a} DS_1 S_2 \xrightarrow{K_c} S_1 DS_2 \]
Figure 11
APPENDIX

This appendix describes the derivation of the various receptor state models and the estimation of the allosteric parameters of nonlabeled ligands measured in competition experiments with labeled ligand and allosteric modulator.

_**Simple two-state model:**_ The affinity constants for the simple two-state model at the third level of analysis are described in Figure 1c. These constants are expressed in inverse molarity units and are defined as the ratio of the ligand-receptor complex divided by the product of the concentrations of ligand and the corresponding receptor state. For example $K_a = [DR]/[D][R]$. The unimolecular constant describing the equilibrium between the free states of the receptor states is defined as: $K_i = R^*/R$.

Fractional receptor occupancy in the presence of allosteric modulator expressed as agonist bound relative to the total receptor concentration ($R_T$) is defined as:

$$\frac{\text{bound}}{R_T} = \frac{DR + DR^* + DRA + DR^*A}{R + R^* + DR + DR^* + RA + R^*A + DRA + DR^*A} \quad (11)$$

It is possible to replace each term in the numerator and denominator on the right hand side of equation 10 with a product of affinity constants and $R$. For example:

$$DR^* = DK_bK,R \quad (12)$$

Using a similar strategy for the other terms, equation 10 can be rewritten as:

$$\frac{\text{bound}}{R_T} = \frac{DK_a,R + DK_bK,R + ADK_aK_cR + ADK_bK_fK,R}{R + K,R + DK_a,R + DK_bK_fR + AK_cR + AK_bK_fR + ADK_aK_cR + ADK_bK_fK,R} \quad (13)$$

Factoring out $R$ and simplifying yields:

$$\frac{\text{bound}}{R_T} = \frac{D}{D + K_{obs}} \quad (14)$$

in which $K_{obs}$ can be defined in terms of third level parameters (Ehlert, 2000):

$$K_{obs} = \frac{1 + K_c + AK_c + AK_bK_i}{K_a + K_bK_i + AK_cK_i + AK_bK_fK_i} \quad (15)$$
As described previously (Ehlert, 1988a), $K_{obs}$ can also be defined in terms of second level parameters:

$$K_{obs} = \frac{1 + AK_2}{K_1 + A\alpha K_1 K_2}$$

Equation 16 can be rearranged into the following form illustrating that the allosteric modulator has a multiplicative effect on the observed affinity of the orthosteric ligand complex corresponding to the factor $p$:

$$K_{obs} = p \frac{1}{K_1}$$

in which $p$ is defined as:

$$p = \frac{1 + AK_2}{1 + A\alpha K_2}$$

The foregoing second level parameters can be defined in terms of third level parameters (Ehlert, 2000):

$$K_1 = \frac{K_a + K_b K_i}{1 + K_i}$$

$$K_2 = \frac{K_e + K_f K_i}{1 + K_i}$$

$$\alpha = \frac{(1 + K_i)(K_a K_e + K_b K_f K_i)}{(K_e + K_f K_i)(K_a + K_b K_i)}$$

The total proportion of receptors in the active state ($R_{active}/R_T$) can be described as:

$$\frac{R_{active}}{R_T} = \frac{R^* + R^* A + DR^* + DR^* A}{R + R^* + DR + DR^* + RA + R^* A + DRA + DR^* A}$$

Using a strategy similar to that described above for equation 11, it is possible to reduce equation 22 to the following (Ehlert, 2000):

$$\frac{R_{active}}{R_T} = \frac{1}{1 + \frac{1 + DK_f + AK_f + AK_b K_f}{K_i(1 + DK_b + AK_f + ADK_b K_f)}}$$

It is useful to consider the proportion of orthosteric ligand-receptor complex in the active conformation ($DR_{active}/R_T$):

$$\frac{DR_{active}}{R_T} = \frac{DR^* + DR^* A}{R + R^* + DR + DR^* + RA + R^* A + DRA + DR^* A}$$
This equation represents the orthosteric ligand-induced change in activation of the receptor from basal activity. Using a strategy similar to that described above, it is possible to reduce equation 24 to the following:

\[
\frac{DR_{\text{active}}}{R_f} = \frac{1}{1 + \frac{1}{K_i} + \frac{1}{K_a} + \frac{AK_e + AK_f + ADK_eK_i}{DK_iK_e + ADK_iK_fK_e}}
\]

In the absence of the allosteric modulator (A = 0), equation 25 reduces to:

\[
\frac{DR_{\text{active}}}{R_f} = \frac{1}{1 + \frac{1}{K_i} + \frac{1}{DK_i}}
\]

The maximum of this function represents the maximum stimulus or observed intrinsic efficacy (e) elicited by the orthosteric ligand, and it can be derived by taking its limit as D approaches infinity:

\[
e = \frac{1}{1 + \frac{K_a}{K_bK_i}}
\]

By setting equation 25 equal to one half times equation 27 and solving for D, it is possible to show that the concentration of D required for half maximal receptor activation is equivalent to K_{obs} (see equation 15) in the absence of A. The equation describing receptor activation in the presence of a maximally effective concentration of the allosteric modulator can be derived by taking the limit of equation 25 as A approaches infinity:

\[
\frac{DR_{\text{active}}}{R_f} = \frac{1}{1 + \frac{K_e}{K_f} + \frac{DK_eK_fK_i}{DK_iK_fK_i}}
\]

The maximum of this function represents the maximum stimulus or the observed intrinsic efficacy of the orthosteric ligand in the presence of a maximally effective concentration of the allosteric modulator (e') and can be derived by taking its limit as D approaches infinity:

\[
e' = \frac{1}{1 + \frac{K_aK_e}{K_bK_fK_i}}
\]

By setting equation 25 equal to one half times equation 29 and solving for D, it is possible to show that the concentration of D required for half maximal receptor activation in the
presence of a maximally effective concentration of $A$ is equivalent to $K_{obs}$ (see equation 15). The parameter $\beta$ is defined as the efficacy of the orthosteric ligand-receptor-allosteric ligand complex ($\epsilon'$) complex expressed relative to that of the orthosteric ligand-receptor complex ($\epsilon$) (Ehlert, 1988a; Ehlert, 2005). This parameter can be expressed in microscopic constants by taking the ratio of $\epsilon'$ to $\epsilon$:

$$\beta = \frac{\epsilon'}{\epsilon} = \frac{1}{1 + \frac{K_a K_e}{K_b K_i} + \frac{K_a}{K_b K_i}}$$

This equation reduces to:

$$\beta = \frac{K_a K_f + K_b K_i}{K_a K_e + K_b K_i}$$

The product of the maximal change in observed affinity ($\alpha$) and intrinsic efficacy ($\beta$) caused by the allosteric ligand can be derived by taking the product of equations 21 and 31, which yields:

$$\alpha \beta = \frac{K_f + K_f K_i}{K_e + K_f K_i}$$

For a receptor lacking substantial constitutive activity ($K_i \ll 1$), equation 32 reduces to the following for a negative allosteric modulator ($K_f < K_e$):

$$\alpha \beta \approx \frac{K_f}{K_e}$$

Equation 33 will also hold for a positive modulator provided that the selectivity of the modulator for the active state is much less than the reciprocal of the fraction of the free receptor in the active state (i.e., if $K_f/K_e < 1/K_i$).

**Complex two-state model**: The affinity constants for the complex two-state model at the third level of analysis are described in Figure 10. These are expressed in inverse molarity units and are defined as the ratio of the ligand-receptor complex divided by the product of the concentrations of ligand and the corresponding receptor state. For example $K_a = [DR_1]/[D][R_1]$. The
unimolecular constants describing the equilibrium between the free states of the receptor states are defined as: \( K_i = R_i^* / R_i, \quad K_j = R_j / R_j \) and \( K_k = R_k^* / R_k \).

Receptor occupancy by the agonist in the presence of the allosteric ligand can be expressed as:

\[
\frac{\text{bound}}{R_i} = \frac{DR_j + DR_j^* +DR_jA + DR_j^* A + DR_k + DR_k^* A}{R_i + R_j + R_j^* +R_i^* +DR_i + DR_i^* +R_jA + R_i^* A + DR_iA + DR_jA + DR_kA + DR_j^* A}
\]

Using a strategy analogous to that used in equation 11 yields a simple one-site model for agonist receptor occupancy in the presence of allosteric modulator. This model is equivalent to equation 14 with an observed dissociation constant defined in third level parameters as:

\[
K_{obs} = 1 + K_i + K_j (1 + K_k) + A(K_e + K_j K_i + K_j (K_g + K_j K_k))
\]

The observed dissociation constant \( K_{obs} \) can also be defined in terms of second level parameters as shown above in equations 16 - 18. In the case of the complex two-state model however, the second level parameters are defined by the following third level parameters:

\[
K_1 = \frac{K_a + K_h K_i + K_j (K_g + K_j K_k)}{1 + K_i + K_j (1 + K_k)}
\]

\[
K_2 = \frac{K_e + K_j K_i + K_j (K_g + K_h K_k)}{1 + K_i + K_j (1 + K_k)}
\]

\[
\alpha = \frac{(1 + K_i + K_j (1 + K_k)) (K_a K_e + K_h K_j K_i + K_j (K_g + K_h K_k))}{(K_e + K_j K_i + K_j (K_g + K_h K_k)) (K_a + K_j K_i + K_j (K_e + K_h K_k))}
\]

The proportion of receptors in the active state can be described as:

\[
\frac{R_{active}}{R_i} = \frac{R_i^* + R_j^* +R_i^* A + R_j^* A + DR_i^* +DR_i^* A + DR_j^* A + DR_kA + DR_j^* A}{R_i + R_j + R_j^* +DR_i + DR_j^* +R_jA + R_i^* A + DR_iA + DR_jA + DR_kA + DR_j^* A}
\]

Using a strategy analogous to that used with equation 22 yields:

\[
\frac{R_{active}}{R_i} = \frac{1}{1 + K_j + D(K_a + K_j K_i) + A(K_e + K_g K_j) + D Where\text{A}(K_a K_e + K_g K_j K_k) + D Where\text{A}(K_j K_i + K_h K_j K_k) + D Where\text{A}(K_j K_i + K_h K_j K_k)}
\]
Using a strategy similar to that described in equation 24, it is possible to derive the proportion of orthosteric ligand-receptor complex in the active conformation ($DR_{\text{active}}/R_T$):

$$\frac{DR_{\text{active}}}{R_T} = \frac{1}{1 + \frac{K_i + K_j + K_j K_k + D(K_a + K_c K_j) + A(K_c + K_i K_j + K_h K_j K_k) + DA(K_a K_c + K_c K_j K_k)}{D(K_b K_i + K_d K_j K_k + A(K_b K_i + K_a K_h K_j K_k)}}$$

The maximum of this function represents the maximum stimulus elicited by the orthosteric ligand, and it can be derived by taking its limit as $D$ approaches infinity. In the absence of the allosteric modulator ($A = 0$), the maximum denotes the observed intrinsic efficacy ($\varepsilon$) of the orthosteric ligand:

$$\varepsilon = \frac{1}{1 + \frac{K_a + K_c K_j}{K_i K_j + K_j K_k}}$$

In the presence of a maximally effective concentration of the allosteric modulator, the maximum of equation 41 represents the observed intrinsic efficacy of the orthosteric ligand in the presence of a maximally effective concentration of the allosteric modulator ($\varepsilon'$):

$$\varepsilon' = \frac{1}{1 + \frac{K_a K_c + K_c K_j K_k}{K_b K_i + K_a K_h K_j K_k}}$$

Using a strategy like that used with equation 30, it can be shown that the cooperativity constant for the maximal change in efficacy ($\beta$) caused by the allosteric ligand is defined as:

$$\beta = \frac{\varepsilon'}{\varepsilon} = \frac{\left(\frac{K_b K_f K_i + K_d K_h K_i K_k}{K_b K_i + K_d K_j K_k}\left(K_a + K_c K_j + K_h K_j K_k\right)\right)}{\left(\frac{K_a K_c + K_c K_j K_k}{K_b K_i + K_d K_h K_j K_k}\right)}$$

The product of the maximal change in observed affinity ($\alpha$) and intrinsic efficacy ($\beta$) caused by the allosteric ligand can be derived by taking the product of equations 38 and 44, which yields:

$$\alpha \beta = \frac{\left(\frac{K_b K_f K_i + K_d K_h K_i K_k}{K_b K_i + K_d K_j K_k}\left(1 + K_i + K_j + K_k\right)\right)}{\left(\frac{K_a K_c + K_c K_j K_k}{K_b K_i + K_d K_h K_j K_k}\right)}$$

When the equilibrium between the free forms of the receptor favors the $R_I$ state over $R_2$ (i.e., $K_i \gg K_j$ and $K_k$) and the receptor lacks constitutive activity ($K_i << 1$), equation 45 reduces to the following for a negative allosteric modulator ($K_f < K_c$):
Equation 46 will also hold for a positive modulator provided that the selectivity of the modulator for the active state of \( R_1 \) is much less than the reciprocal of the fraction of free \( R_1 \) in the active state (i.e., if \( K_f/K_e < 1/K_i \)). When the equilibrium between the free forms of the receptor favors the \( R_2 \) state over \( R_1 \) (i.e., \( K_j \gg K_i \)) and the receptor lacks constitutive activity (\( K_k << 1 \)), equation 45 reduces to the following for a negative allosteric modulator (\( K_h < K_g \)):

\[
\alpha\beta \approx \frac{K_h}{K_g}
\]

Equation 47 holds for a positive modulator provided that the selectivity of the modulator for the active state of \( R_2 \) is much less than the reciprocal of the fraction of free \( R_2 \) in the active state (i.e., if \( K_h/K_g < 1/K_k \)). It can be seen from equations 46 and 47 that the value of the product \( \alpha\beta \) is bounded by the interval of \( K_f/K_e \) to \( K_h/K_g \).

**Tandem two-site, two-state model:** The affinity constants for the tandem, two-site, two-state model at the third level of analysis are described in Figure 10c. These are expressed in inverse molarity units and are defined as the ratio of the ligand-receptor complex divided by the product of the concentrations of ligand and the corresponding receptor state. For example \( K_a = [DS_1S_2]/[D][S_1S_2] \). The unimolecular constants describing the transfer of ligand between the relay site and the ground and active states of the activation site are defined as: \( K_c = S_1DS_2/[DS_1S_2] \) and \( K_d = S_1DS_2*/[DS_1S_2] \). The equilibrium between the free states of the relay site and the activation site are independent of each other and are defined as: \( K_g = S_1'/S_1 \) and \( K_h = S_2'/S_2 \).

The amount of bound orthosteric ligand in the presence of allosteric modulator is defined as:

\[
\text{bound} = [DS_1S_2] + [DS_1'S_2] + [DS_1S_2*] + [DS_1'S_2*] + [S_1DS_2] + [S_1'DS_2] + [S_1DS_2*] + [S_1'DS_2*] + [DS_1AS_2] + [DS_1'AS_2] + [DS_1AS_2*] + [DS_1'AS_2*] + [S_1ADS_2] + [S_1'ADS_2] + [S_1ADS_2*] + [S_1'ADS_2*]
\]

The total amount of receptor is defined as:
\[
R_T = [S_iS_j] + [S_i'S_j] + [S_iS_j^*] + [S_iA_S_j] + [S_i'AS_j] + [S_iA_S_j^*] + [DS_iS_j] +
[DS_i'S_j] + [DS_iS_j^*] + [DS_iDS_j] + [S_i' DS_j] + [S_iDS_j^*] + [S_iDS_j] + [DS_iAS_j] +
[DS_i'AS_j] + [DS_iAS_j^*] + [S_iADS_j] + [S_i'ADS_j] + [S_iADS_j^*] + [S_i'ADS_j]  
\]

Using a strategy analogous to that used in equation 11 yields a simple one-site model for agonist receptor occupancy in the presence of allosteric modulator. This model is equivalent to equation 14 with an observed dissociation constant defined as:

\[
K_{obs} = \frac{(1 + K_h)(1 + K_g + A(K_e + K_fK_g))}{(1 + K_e + K_h + K_dK_h)(K_a + K_bK_g + A(K_aK_e + K_bK_fK_g))}  
\]

This \( K_{obs} \) can also be defined in second level parameters:

\[
K_{obs} = \frac{1}{K_{1-R} + K_{1-A}K_{1-A} + A\alpha K_{1-R}K_2 + A\alpha K_{1-A}K_1K_2}  
\]

Equation 51 can be rearranged into the following form illustrating that the allosteric modulator has a multiplicative effect on the observed affinity of the orthosteric ligand complex corresponding to the factor \( p \):

\[
K_{obs} = p \frac{1}{K_{1-R} + K_{1-A}K_{1-A}}  
\]

in which \( p \) is defined as:

\[
p = \frac{1 + AK_2}{1 + A\alpha K_2}  
\]

The foregoing second level parameters can be defined in terms of third level parameters:

\[
K_{1-R} = \frac{K_a + K_bK_g}{1 + K_g}  
\]

\[
K_{1-A} = \frac{K_a + K_bK_h}{1 + K_h}  
\]

\[
K_2 = \frac{K_e + K_fK_g}{1 + K_g}  
\]

\[
\alpha = \alpha_R = \frac{(K_aK_e + K_bK_fK_g)(1 + K_g)}{(K_e + K_fK_g)(K_a + K_bK_g)}  
\]

The cooperativity constant \( \alpha_R \) denotes the cooperativity between the binding of \( D \) and \( A \) at the relay site. It can be shown that whenever the constant describing the transfer of \( D \) from the relay site to the activation site is independent of the conformation of the relay site, then \( \alpha_R = \alpha \), where \( \alpha \) denotes the observed cooperativity constant in the allosteric ternary complex model (Figure
1b). In other words, if $DS_1S_2/S_1DS_2 = DS_1'S_2/S_1'DS_2 = K_c$ and $DS_1S_2*/S_1DS_2* = DS_1'S_2*/S_1'DS_2*$ = $K_d$, then $\alpha_R = \alpha$. We use different variables to denote $\alpha_R$ and $\alpha$ because a modification of the tandem two-site, two-state model without the forgoing constraint would yield different values for $\alpha_R$ and $\alpha$.

The amount of receptors in the active state is defined as:

$$ R_{active} = [S_1S_2*] + [S_1'AS_2*] + [S_1'S_2*] + [DS_1S_2*] + [DS_1'S_2*] + [S_1DS_2*] + [S_1'DS_2*] + [DS_1AS_2*] + [DS_1'AS_2*] + [S_1ADS_2*] + [S_1'ADS_2*] $$

The total amount of receptors ($R_T$) is defined in equation 49. Using a strategy analogous to that used in equation 11 yields the equation for the amount of receptor in the active state:

$$ \frac{R_{active}}{R_T} = \frac{1}{1 + K_{active}} $$

in which,

$$ K_{active} = \frac{\text{numerator}_1}{\text{denominator}_1} $$

$$ \text{numerator}_1 = 1 + K_g + D(K_a + K_oK_c + K_bK_g + K_bK_cK_g) + A(K_e + K_fK_g) + DA(K_aK_e + K_oK_cK_e + K_bK_fK_g + K_bK_dK_g) $$

$$ \text{denominator}_1 = K_h((1 + K_g) + D(K_a + K_oK_c + K_bK_g + K_oK_d) + A(K_e + K_fK_g) + DA(K_aK_e + K_oK_c + K_bK_fK_g + K_bK_dK_g)) $$

The proportion of orthosteric ligand-receptor complex in the active state ($DR_{active}/R_T$) is defined as:

$$ \frac{DR_{active}}{R_T} = \frac{1}{1 + K_{DR-active}} $$

in which,

$$ K_{DR-active} = \frac{\text{numerator}_2}{\text{denominator}_2} $$

$$ \text{numerator}_2 = 1 + K_g + K_h + K_gK_h + D(K_a + K_bK_g)(1 + K_c + K_h) + A(K_e + K_fK_g)(1 + K_h) + AD(K_aK_e + K_bK_fK_g)(1 + K_e + K_h) $$

$$ \text{denominator}_2 = DK_aK_b((K_a + K_bK_g) + A(K_aK_c + K_bK_fK_g)) $$
By setting \( A = 0 \) in equation 63 and taking its limit as \( D \) approaches infinity, it is possible to derive the maximum stimulus (\( \varepsilon \)) generated by the orthosteric ligand in the absence of the allosteric modulator:

\[
\varepsilon = \frac{1}{1 + \frac{1 + K_c + K_h}{K_d K_h}} 
\]  

Since \( K_c \) denotes the unimolecular constant describing the transfer of orthosteric ligand from the relay site (S_1) to the inactive state of the activation site (S_2), we would expect that \( K_c > 1 \). Thus, for a receptor lacking constitutive activity (\( K_h \ll 1 \)), observed intrinsic efficacy can be well approximated by:

\[
\varepsilon \approx \frac{1}{1 + \frac{K_c}{K_d K_h}} 
\]  

By taking the limit of equation 63 as both \( A \) and \( D \) approach infinity, it is possible to estimate the maximum stimulus generated by the orthosteric ligand in the presence of a maximally effective concentration of the allosteric modulator (\( \varepsilon' \)):

\[
\varepsilon' = \frac{1}{1 + \frac{1 + K_c + K_h}{K_d K_h}} 
\]  

It can be seen that the observed intrinsic efficacy of the orthosteric ligand in the absence of the allosteric modulator (equation 67) is the same as that in the presence of the modulator (equation 69), indicating that the allosteric modulator has no effect on the observed intrinsic efficacy of the orthosteric ligand receptor complex:

\[
\beta = \frac{\varepsilon'}{\varepsilon} = 1
\]  

**Analysis of agonist/[\(^3\)H]NMS competition experiments in the presence of gallamine:** The scheme describing the competitive interaction between agonist (\( D \)) and [\(^3\)H]NMS (\( L \)) at the M_2 muscarinic receptor in the presence of gallamine (\( A \)) is described as (Ehlert, 1985):
The various affinity constant are defined above and subscripts have been added to discriminate between the parameters of the agonist and [³H]NMS. The analytical solution to this scheme of equilibria has been described previously within the context of a ternary complex model (Ehlert, 1985):

\[
\text{bound} = LR + LRA = \frac{L \times R_T}{L + K_{obs}}
\]

in which

\[
K_{obs} = \frac{1 + DK_{1-D} + AK_2(1 + D\alpha_{D}K_{1-D})}{K_{1-NMS} + A\alpha_{NMS}K_{1-NMS}K_2}
\]

In the absence of agonist \((D = 0)\), the observed dissociation constant of [³H]NMS reduces to:

\[
K_{obs}' = \frac{1 + AK_2}{K_{1-NMS} + A\alpha_{NMS}K_{1-NMS}K_2}
\]

in which \(K_{obs}'\) denotes the observed dissociation constant of [³H]NMS in the absence of agonist.

The equation describing the competitive inhibition of the binding of a fixed concentration of [³H]NMS by various concentration of agonist in the presence of fixed concentration of gallamine can be derived by taking the ratio of equation 71 divided by the same equation with \(K_{obs}'\) substituted in for \(K_{obs}\).

\[
F = \frac{L \times R_T}{L + K_{obs}} = \frac{L + K_{obs}'}{L + K_{obs}}
\]

In this equation, \(F\) denotes the fractional binding of [³H]NMS at a fixed concentration measured in the presence of various concentrations of agonist and a fixed concentration of gallamine divided by that measured in the absence of agonist. Substituting in equations 72 and 73 for \(K_{obs}\) and \(K_{obs}'\) followed by rearrangement yields:
When $D$ is present at a concentration causing 50% displacement specific [^3]H]NMS binding, equation 75 becomes:

$$ F = \frac{1}{1 + \frac{D(K_{1-D} + A\alpha_D K_{1-D} K_2)}{1 + AK_2 + L(K_{1-NMS} + A\alpha_{NMS} K_{1-D} K_2)}} $$

When $D$ is present at a concentration causing 50% displacement specific [^3]H]NMS binding, equation 75 becomes:

$$ \frac{1}{2} = \frac{1}{1 + \frac{IC_{50} ' (K_{1-D} + A\alpha_D K_{1-D} K_2)}{1 + AK_2 + L(K_{1-NMS} + A\alpha_{NMS} K_{1-D} K_2)}} $$

in which $IC_{50} '$ denotes the $IC_{50}$ value of the agonist measured in the presence of gallamine. Solving this equation for $IC_{50} '$ yields:

$$ IC_{50} ' = \frac{1 + AK_2 + L(K_{1-NMS} + A\alpha_{NMS} K_{1-NMS} K_2)}{K_{1-D} + A\alpha_D K_{1-D} K_2} $$

Equation 77 can be used to describe the $IC_{50}$ value of the agonist in the absence of gallamine by setting $A = 0$. Under this condition, equation 77 reduces to:

$$ IC_{50} = \frac{1 + LK_{1-NMS}}{K_{1-D}} $$

Equation 78 is equivalent to that described by Cheng and Prusoff (1973), with the dissociation constants replaced with affinity constants. The shift in the agonist/[^3]H]NMS competition curves caused by gallamine can be derived by taking the ratio of the $IC_{50}$ value of the agonist measured in the presence of gallamine divided by that measured in its absence ($R$):

$$ R = \frac{IC_{50} '}{IC_{50}} $$

Substituting in equations 77 and 78 for $IC_{50} '$ and $IC_{50}$ followed by rearrangement yields:

$$ R = \frac{1 + AK_2 + L(K_{1-NMS} + A\alpha_{NMS} K_{1-NMS} K_2)}{1 + LK_{1-NMS} + A\alpha_D K_2 + AL\alpha_D K_{1-NMS} K_2} $$

Solving this equation for $\alpha_D$ yields equation 7 under “Materials and Methods.”