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Dr. Petrus Johan Pauwels, Editor
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Dear Dr. Pauwels,

We are submitting a manuscript, entitled “Analysis of ligand bias in functional studies involving the allosteric modulation of G protein-coupled receptors” for your consideration for publication in *Journal of Pharmacological and Toxicological Methods*.

We look forward to your editorial comments.

Sincerely,

Frederick J. Ehlert, Ph.D.
Professor

Analysis of Ligand Bias in Functional Studies Involving the Allosteric Modulation of G Protein-Coupled Receptors

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Abbreviations

4-DAMP mustard, N-(2-chloroethyl)-4-piperidinyl diphenylacetate; BQCA, 1-(4-Methoxybenzyl)-4-oxo-1,4-dihydro-3-quinoline carboxylic acid; GPCR, G protein-coupled receptor.

Abstract

Introduction: The affinity constants a ligand for active and inactive states of a receptor ultimately determine its capacity to activate downstream signaling events. In this report, we describe a reverse-engineering strategy for estimating these microscopic constants.

Methods: Our approach involves analyzing responses measured downstream in the signaling pathway of a G protein-coupled receptor under conditions of allosteric modulation and reduced receptor expression or partial receptor inactivation. The analysis also yields estimates of the isomerization constant of the unoccupied receptor, the sensitivity constant of the signaling pathway, and the more empirical parameters of the receptor population including the observed affinities and efficacies of allosteric and orthosteric ligands – including inverse agonists – and the efficacy of the unoccupied receptor (i.e., constitutive activity).

Results and Discussion: We validate our approach with an analytical proof and by analysis of simulated data. We also use our method to analyze data from the literature. We show that the values of the microscopic constants of orthosteric and allosteric ligands are constant regardless of the allosteric interaction and the nature of the receptor-signaling pathway as long as the same active state mediates the response. Our analysis is useful for quantifying probe-dependent allosteric interactions and the selectivity of agonists for different signaling pathways. Knowing the isomerization constant and sensitivity constant of a signaling pathway in a given cell line or tissue preparation enables future investigators to estimate the affinity constants of agonists for receptor states simply through analysis of their concentration-response curves. Our approach also provides a means of validating *in silico* estimates of ligand affinity for crystal structures of active and inactive states of the receptor.

1. Introduction

Scientists are often interested in how well an agonist activates a specific G protein-coupled receptor (GPCR). Activation is usually assessed by measuring a response downstream in the signaling pathway, like heart rate, cAMP accumulation, phosphoinositide hydrolysis, mobilization of Ca^{2+}, contraction of smooth muscle, or recruitment of arrestin. Depending on which response is measured, however, the potency and maximal response of a given agonist can vary substantially because of differences in downstream signaling machinery. The same can be said of allosteric interactions with the added complication that the modulation varies depending on the orthosteric ligand participating in the interaction (Valant, Felder, Sexton, & Christopoulos, 2012). How then do we to assess drug-receptor interactions in a way that is unaffected by downstream signaling events and the interacting ligands?

In the case of ligand-gated ion channels, the activation state of the receptor population can be measured directly as the whole-cell current response under voltage clamp conditions. The analogous measurement for a population of GPCRs (i.e., amount of receptor in the active state in a complex with GDP-bound G protein) is difficult to achieve, but it can be deduced by reverse engineering (Black & Leff, 1983) or response-clamp analysis (Furchgott & Bursztyn, 1967) of a set of responses measured downstream in the signaling pathway under control conditions and after inactivation of a portion of the receptor population. These analyses yield estimates of the observed affinity constant (K_{obs}) of the agonist-receptor complex and a relative measure of the fraction of the occupied receptor population in the active state (ε, efficacy). But for a given agonist-GPCR pair, these population parameters vary, depending on the G protein (Kenakin, 2011) and the concentration of GTP (F. J. Ehlert, 2000; F. J. Ehlert & Rathbun, 1990).

At a deeper level of analysis, drug-receptor interactions are described in terms of affinity constants for active and inactive states of the receptor (Colquhoun & Hawkes, 1982; Monod, Wyman, & Changeux, 1965). These are the ultimate determinants of agonist action because the active state is the first cause of more distal responses.

Colquhoun and Hawkes (1982) developed a method for estimating the transition rate constants for open and closed states of ligand-gated ion channels. Their approach involves analyzing single-channel events as a continuous Markov process within the constraints of a two-state receptor scheme. Using a similar analysis and equilibrium relationships, Auerbach and coworkers (1999; 2010) have estimated the affinity constants of acetylcholine for open (J_D, 5 x 10^7) and closed (K_D, 7.1 x 10^3 M^{-1}) states of the muscle-type nicotinic acetylcholine receptor.

The affinity constant of a ligand for the active state of a GPCR (K_b, see Figure 1) is related to the population parameters (K_{obs} and ε) of the agonist-receptor complex. For example, the product of affinity and efficacy ($K_{obs}\varepsilon$) is proportional to K_b (Tran, Chang, Matsui, & Ehlert, 2009), and the proportionality constant is related to constitutive activity (F. J. Ehlert, Suga, & Griffin, 2011a). Thus, both relative (K_b of one agonist relative to that of another, RA_i) and absolute estimates of K_b (i.e., in units of M^{-1}) can be determined from downstream responses depending on whether constitutive activity can be measured. Reasonable estimates of the affinity constant of the inactive state (K_a) can be calculated for all but the most efficacious agonist in a series (F. J. Ehlert, Griffin, & Suga, 2011).

If the effects of a range of concentrations of allosteric ligand on the concentration-response curve of an agonist for eliciting a response through a GPCR are measured, then the observed affinity of the allosteric ligand and the product of its scalar effects on the observed affinity (α) and efficacy (β_1) of the orthosteric ligand ($\gamma_1 = \alpha\beta_1$) can be estimated (F. J. Ehlert, 1988a, 2005). In many instances, the values of K_{obs} and γ_1 are nearly equivalent to the affinity constant of the allosteric ligand for the inactive state of the receptor (K_e) and the ratio of affinity constants of the active and inactive states (K_f/K_e), respectively (F. J. Ehlert & Griffin, 2008). To our knowledge, there are no reports describing a robust method for estimating both the affinity (α) and efficacy (β_1) components of allosteric modulation in functional assays on GPCRs without also incorporating a measurement of K_{obs} from another source (e.g., binding experiment).

A useful but difficult parameter to estimate is the isomerization constant of the unoccupied receptor (E_o). This parameter is defined as the ratio of concentrations of active (R_s^*) and inactive

(R_s) states of unoccupied receptor (i.e., $E_o = [R_s^*]/[R_s]$), and it determines how much spontaneous receptor activation occurs in the absence of ligands. By examining how point mutations that cause spontaneous channel opening affect acetylcholine-induced currents, Auerbach and coworkers (2012; 2009) estimated the E_o value of the unoccupied muscle-type nicotinic acetylcholine receptor to be approximately 7×10^{-7}. Similar values were estimated by Jackson (2012) and Neubig and Cohen (1980). To our knowledge, no analogous estimate has been made for GPCRs. Point mutations that cause constitutive activity increase the isomerization constant of the unoccupied receptor, and positive allosteric ligands have an analogous effect. Thus, the analysis of allosteric interactions under the appropriate conditions should yield estimates of the isomerization constant of the unoccupied receptor.

Here, we describe the theory and conditions for estimating of all of the population parameters for allosteric interactions at GPCRs. We show that when the interaction is consistent with a two-state scheme, it is also possible to estimate the microscopic constants (K_a, K_b, K_e, and K_f) of the interacting ligands as well as the isomerization constant of the unoccupied receptor ($K_{q\text{-}obs}$), the sensitivity constant of the signaling pathway ($K_{E\text{-}obs}$), the efficacies of orthosteric agonists and inverse agonists (ε) and of allosteric ligands (ε_A), and the activity of the unoccupied receptor complex (constitutive activity, ε_{sys}). We demonstrate our approach using simulated data and data from the literature, and we also provide an analytical proof for the validity of our approach. Knowing the values of $K_{E\text{-}obs}$ and $K_{q\text{-}obs}$ for a specific receptor-signaling pathway or response enables the estimation of the K_a and K_b values of any agonist through analysis of its concentration-response curve. Our method will enable investigators to estimate the microscopic constants of endogenous ligands and other orthosteric agonists and inverse agonists and enable quantification of probe-dependent allosteric interactions and the selectivity of agonists for different signaling pathways.

2. Methods

We begin by describing the theoretical basis for the analysis of allosteric interactions in functional assays so that the affinity constants of the interacting ligands for active and inactive states of the receptor can be estimated. We also describe the requisite theory for estimation of the observed parameters of the receptor population. This process involves developing the appropriate equations for global nonlinear regression analysis of functional data. To assess the feasibility of our approach we simulate data using the model shown in Figure 1 and analyze these data by our regression equations to determine if the parameters used to simulate the data can be estimated. These four steps are described in the following sections entitled, "Analysis of data, receptor states", "Analysis of data, parameters of the receptor population", "Simulation of agonist concentration-response curves" and "Nonlinear regression analysis."

2.1 Analysis of data, receptor states

The output of the scheme in Figure 1 is consistent with a simple one-site model with constitutive activity as described below in connection with Figure 3. Thus, the simple allosteric two-state scheme shown in Figure 2*a* adequately describes the output. This scheme is equivalent to that described by Monod Wyman and Changeux (1965), but it also includes a conformational induction step ($DR_s \leftrightarrow DR_s^*$). The scheme can be solved for the fractional amount of receptor in the active state including both constitutive (R_s^*) and ligand-activated ($DR_s^* + R_s^*A + DR_s^*A$) forms as described previously (F. J. Ehlert & Griffin, 2008):

$$T = \frac{[R_S^*]+[DR_S^*]+[R_S^*A]+[DR_S^*A]}{[R_T]} = \frac{1}{1+\frac{(DK_a+1)(AK_e+1)}{K_{q-obs}(DK_b+1)(AK_f+1)}} \qquad 1$$

In this equation, R_T denotes the total amount of receptor, K_b and K_a the microscopic affinity constants (inverse molar units, M^{-1}) of the orthosteric ligand for the active and inactive states, K_f and K_e represent the corresponding parameters for the allosteric ligand and $K_{q\text{-}obs}$ denotes the observed isomerization constant of the unoccupied receptor. As described below, the value of

$K_{q\text{-}obs}$ is perturbed from K_q by endogenous G protein and guanine nucleotide. Equation 1 was substituted into the transducer function of Black and Leff (1983)

$$response = \frac{M_{sys}T^m}{T^m + K_E^m} \qquad 2$$

to yield an equation for the downstream response expressed as a function of the microscopic constants of the allosteric model (Figure 2*a*) as described previously (F. J. Ehlert & Griffin, 2008).

$$response = \frac{M_{sys}}{1 + K_{E-obs}^m \left(1 + \frac{(DK_a+1)(AK_e+1)}{K_{q-obs}(DK_b+1)(AK_f+1)}\right)^m} \qquad 3$$

In this equation, M_{sys} represents the maximum response of the system, m, the transducer slope factor and $K_{E\text{-}obs}$, the observed sensitivity constant of the operational model. As described below, the value of $K_{E\text{-}obs}$ differs from K_E in the operational model (see equation 2) because of the influence of G protein and guanine nucleotides.

In many instances, it is impossible to estimate the individual parameters, K_b, $K_{q\text{-}obs}$ and $K_{E\text{-}obs}$, but it is possible to estimate the composite parameter, K_{bqs}:

$$K_{bqs} = \frac{K_b K_q}{(1+K_q)K_{E-obs}} \qquad 4$$

Thus, the following form of equation 3 is useful for estimating K_{bqs}:

$$response = \frac{M_{sys}}{1 + K_{E-obs}^m \left(1 + \frac{(DK_a+1)(AK_e+1)}{K_{q-obs}\left(\frac{DK_{bqs}(1+K_{q-obs})K_{E-obs}}{K_{q-obs}}+1\right)(AK_f+1)}\right)^m} \qquad 5$$

The parameter K_{bqs} is useful for estimating a relative value of K_b (see equation 15).

More parameters can be estimated if some of the data are measured following inactivation of a fraction of the orthosteric binding sites with a ligand that behaves as a neutral antagonist when bound irreversibly. Under this condition, the receptor population behaves as two subpopulations – one unaffected by the irreversible antagonist and the other having its orthosteric sites

irreversibly blocked. The contributions of the two subpopulations are denoted by q and $1 - q$, where q denotes the fraction of the residual orthosteric sites unaffected by the irreversible ligand. The total stimulus (T) after partial receptor inactivation is equivalent to the sum of the two subpopulations:

$$T = \frac{q}{1+\frac{(DK_a+1)(AK_e+1)}{K_{q-obs}(DK_b+1)(AK_f+1)}} + \frac{1-q}{1+\frac{(AK_e+1)}{K_{q-obs}(AK_f+1)}} \qquad 6$$

The fraction on the left describes the stimulus of the residual unalkylated receptors and was derived by multiplying equation 1 by q. The second fraction describes the stimulus of the alkylated receptor subpopulation, and was derived by solving equation 1 under the condition, D = 0, and multiplying the result by $1 - q$. Substituting this equation into the transducer function (equation 2) yields an equation for the response after partial receptor inactivation in terms of the microscopic constants:

$$response = \frac{M_{sys}}{1+K_{E-obs}^{m}\left(\frac{q}{1+\frac{(DK_a+1)(AK_e+1)}{K_{q-obs}(DK_b+1)(AK_f+1)}}+\frac{1-q}{1+\frac{(AK_e+1)}{K_{q-obs}(AK_f+1)}}\right)^{-m}} \qquad 7$$

When there is no constitutive activity and the allosteric ligand lacks activity by itself, it is impossible to estimate K_b and $K_{q\text{-}obs}$ accurately, but it is possible to estimate K_{bqs}. In this situation, the contribution of the alkylated receptor complex is negligible, and hence, a reduced form of equation 7, incorporating the substitution for K_{bqs}, adequately describes the data:

$$response = \frac{M_{sys}}{1+K_{E-obs}^{m}\left(\frac{1}{q}+\frac{(DK_a+1)(AK_e+1)}{q\left(DK_{bqs}K_{E-obs}\left(1+K_{q-obs}\right)+K_{q-obs}\right)\left(AK_f+1\right)}\right)^{m}} \qquad 8$$

For analyzing the condition of no allosteric ligand ($A = 0$), equation 7 reduces to:

$$response = \frac{M_{sys}}{1+K_{E-obs}^{m}\left(\frac{q}{1+\frac{(DK_a+1)}{K_{q-obs}(DK_b+1)}}+\frac{1-q}{1+\frac{1}{K_{q-obs}}}\right)^{-m}} \qquad 9$$

In situations where an irreversible antagonist is unavailable, responses can be measured under the condition of reduced receptor expression. In this case, all active forms of the receptor are reduced by the scalar q, which represents fractional receptor expression relative to control expression. The total stimulus for this condition is derived by multiplying equation 1 by q:

$$T = \frac{q}{1+\frac{(DK_a+1)(AK_e+1)}{K_{q-obs}(DK_b+1)(AK_f+1)}} \qquad 10$$

Substituting this equation for receptor activation into the transducer function (equation 2) yields an equation for the response:

$$response = \frac{M_{sys}}{1+K_{E-obs}^{m}\left(\frac{1}{q}+\frac{(DK_a+1)(AK_e+1)}{qK_{q-obs}(DK_b+1)(AK_f+1)}\right)^{m}} \qquad 11$$

When the receptor lacks constitutive activity and the allosteric ligand lacks an effect by itself, it is impossible to estimate K_b and $K_{q\text{-}obs}$, but it is possible to estimate K_{bqs} as mentioned above. Substitution of K_{bqs} (equation 4) for K_b in equation 11 yields equation 8. Thus, the latter equation can be used for reduced receptor expression or partial receptor inactivation.

For analyzing the condition of no allosteric ligand ($A = 0$), equation 11 reduces to:

$$response = \frac{M_{sys}}{1+K_{E-obs}^{m}\left(\frac{1}{q}+\frac{(DK_a+1)}{qK_{q-obs}(DK_b+1)}\right)^{m}} \qquad 12$$

If there is only one active state that mediates the response, then each estimate of a microscopic constant for an allosteric (K_e and K_f) or orthosteric (K_a and K_b) ligand should lack a statistically significant difference when estimated from the independent effect of the ligand or from its interaction with other ligands. The appropriate equations, described above, solved for the condition of no allosteric modulator ($A = 0$), can be used for this determination. As long as no interacting ligand is present, the estimates of K_a and K_b represent microscopic affinity constants for active and inactive states, regardless of whether the ligand is orthosteric or allosteric.

If the receptor lacks constitutive activity, it is impossible to estimate the K_b value of an orthosteric ligand from its concentration-response curve in the absence of an allosteric ligand.

Therefore, we developed an analysis for a group of ligand concentration-response curves that yields K_a and a relative estimate of the microscopic affinity constant for the active state (RA_i) under experimental conditions of no constitutive activity. For this analysis, equation 8 is solved for the condition of no allosteric modulator:

$$response = \frac{M_{sys}}{1+K_{E-obs}^{m}\left(\frac{1}{q}+\frac{\left(DK_a'+1\right)}{qDK_{bqs}'K_{E-obs}\left(1+K_{q-obs}\right)+K_{q-obs}}\right)^{m}} \qquad 13$$

In this equation, K_{bqs}' and K_a' denote the K_{bqs} and K_a values of the most efficacious agonist (standard agonist). For analysis of less efficacious agonists (test agonists) and the condition of q = 1, equation 13 can be rearranged into the following form:

$$response = \frac{M_{sys}}{1+K_{E-obs}^{m}\left(1+\frac{\left(DK_a+1\right)}{DK_{bqs}'RA_iK_{E-obs}\left(1+K_{q-obs}\right)+K_{q-obs}}\right)^{m}} \qquad 14$$

in which, RA_i (intrinsic relative activity) denotes the K_{bqs} value of a test agonist expressed relative to that of the standard agonist (K_{bqs}'). Thus, RA_i is a relative measure of the microscopic affinity constant of the active state of the receptor as described previously:

$$RA_i = \frac{K_b}{K_b'} = \frac{K_{bqs}}{K_{bqs}'} \qquad 15$$

In this equation, K_b' denotes the K_b value of the most efficacious agonist.

For implementing this type of analysis, equation 13 is fitted to the concentration-response curves of the standard agonist, measured in the absence and presence of reduced receptor expression or partial receptor inactivation, and equation 14 is fitted to the concentration-response curves of the test agonists. Global nonlinear regression analysis is used, sharing the estimates of K_{bqs}', $K_{E\text{-}obs}$, M_{sys} and m among the data, and obtaining unique estimates of the other parameters for each ligand. The analysis can be done without measuring the concentration-response curve of the standard agonist after partial receptor inactivation, but this omission will prevent the estimation of the K_a values of the test agonists. In this case, equation 13 is simplified by constraining q to one.

2.2 Analysis of data: parameters of the receptor population

The derivation of equations describing the response as a function of the empirical parameters of the receptor population is described in this section. Ultimately, the parameters of the receptor population are estimated by fitting these equations to functional data using nonlinear regression analysis.

The scheme in Figure 2*a* can be described without concern for the state of the receptor as shown in Figure 2*b*. This scheme is known as the allosteric ternary complex model, and it describes the behavior of the receptor population (F. J. Ehlert, 1988a). The parameter K_1 denotes the observed affinity constant (inverse molar units, M^{-1}) of the orthosteric ligand in the absence of the allosteric ligand, and K_2 denotes the corresponding constant for the allosteric ligand. The constant α denotes the scalar change in affinity of each ligand for the ternary complex caused by the binding of the other ligand. The fractional amount of the population of each receptor complex (*R*, *DR*, *RA* and *DRA*) in the active state is defined as observed efficacy (ε_{sys}, ε, ε_A, and $\beta_1\varepsilon$, respectively), and these variables are evaluated relative to the maximal amount of receptor complex in the active state as defined by the variable T_{max} in equation 35. For the case of one orthosteric and one allosteric binding site per receptor complex, each efficacy term is constant for any level of receptor occupancy. The equation for fractional receptor activation (($\varepsilon_{sys} + \varepsilon DR + \varepsilon_A RA + \beta_1\varepsilon DRA)/R_T$) can be solved using an approach analogous to that described previously (F. J. Ehlert & Griffin, 2008):

$$T = \frac{\varepsilon_{sys}+\varepsilon_A AK_2+\varepsilon DK_1(1+A\gamma_1 K_2)}{1+AK_2+DK_1(1+A\alpha K_2)} \qquad 16$$

To derive an equation for the analysis of concentration-response curves the total population stimulus (*T*) from equation 16 is substituted into the transducer function (equation 2):

$$response = \frac{M_{sys}}{1+\left(\frac{1+AK_2+DK_1(1+A\alpha K_2)}{\tau_{sys}+\tau_A AK_2+\tau DK_1(1+A\gamma_1 K_2)}\right)^m} \qquad 17$$

in which,

$$\tau = \frac{\varepsilon}{K_{E-obs}} \quad 18$$

$$\tau_A = \frac{\varepsilon_A}{K_{E-obs}} \quad 19$$

$$\tau_{sys} = \frac{\varepsilon_{sys}}{K_{E-obs}} \quad 20$$

For the condition of no detectable constitutive activity, equation 17 reduces to a form equivalent to that previously described by Gregory and coworkers (2010):

$$response = \frac{M_{sys}}{1+\left(\frac{1+AK_2+DK_1(1+A\alpha K_2)}{\tau_A AK_2+\tau DK_1(1+A\gamma_1 K_2)}\right)^m} \quad 21$$

For the condition of a lack of constitutive receptor activity and an allosteric ligand having no activity by itself, equation 17 reduces to that described previously (F. J. Ehlert, 2005):

$$response = \frac{M_{sys}}{1+\left(\frac{1+AK_2+DK_1(1+A\alpha K_2)}{\tau DK_1(1+A\gamma_1 K_2)}\right)^m} \quad 22$$

In many instances, it is impossible to estimate the individual parameters, K_1 and τ, but it is possible to estimate the composite parameter, τK_1. Thus, the following form of equation 17 is useful for this situation:

$$response = \frac{M_{sys}}{1+\left(\frac{1+AK_2+DK_1(1+A\alpha K_2)}{\tau_{sys}+\tau_A AK_2+D*R(1+A\gamma_1 K_2)}\right)^m} \quad 23$$

In this equation, R denotes the product, τK_1. As described under Appendix (see equation 62), both τK_1 and the composite microscopic parameter, K_{bqs}, are equivalent.

More of the population parameters for allosteric interactions can be estimated if some of the orthosteric sites are inactivated with a ligand that behaves as a neutral antagonist when bound irreversibly. The total stimulus function for this condition represents the sum of the stimuli from the residual unaffected receptors and the receptors whose orthosteric sites have been irreversibly blocked. Their relative contributions are denoted as q and 1- q and are derived analogously to that described above for the receptor state analysis involving receptor inactivation (equation 6):

$$T = \frac{\varepsilon_{sys}+\varepsilon_A AK_2+\varepsilon DK_1(1+A\gamma_1 K_2)}{1+AK_2+DK_1(1+A\alpha K_2)} + (1-q)\frac{\varepsilon_{sys}+\varepsilon_A AK_2}{1+AK_2} \quad 24$$

Substituting equation 24 into the transducer function (equation 2) yields an equation for the response under the condition of partial receptor inactivation:

$$response = \frac{M_{sys}}{1+\left(q\frac{\tau_{sys}+\tau_A AK_2+\tau DK_1(1+A\gamma_1 K_2)}{1+AK_2+DK_1(1+A\alpha K_2)}+(1-q)\frac{\tau_{sys}+\tau_A AK_2}{1+AK_2}\right)^{-m}} \quad 25$$

The corresponding equation for reduced receptor expression is:

$$response = \frac{M_{sys}}{1+\left(q\frac{1+AK_2+DK_1(1+A\alpha K_2)}{\tau_{sys}+\tau_A AK_2+\tau DK_1(1+A\gamma_1 K_2)}\right)^{m}} \quad 26$$

As described below, we analyzed the simulated responses of allosteric and orthosteric ligands independently. For the condition of no allosteric ligand ($A = 0$), equation 25 reduces to (F. J. Ehlert, Suga, et al., 2011a):

$$response = \frac{M_{sys}}{1+\left(q\frac{\tau_{sys}+\tau DK_1}{1+DK_1}+(1-q)\tau_{sys}\right)^{-m}} \quad 27$$

This equation can be rearranged into the following form for the estimation of K_b by taking advantage of equation 115 (Appendix), which describes K_b as a function of τ, K_1 and τ_{sys}:

$$response = \frac{M_{sys}}{1+\left(q\frac{\tau_{sys}+(1+DK_b)}{1+DK_1}+(1-q)\tau_{sys}\right)^{-m}} \quad 28$$

The corresponding equation for reduced receptor expression is:

$$response = \frac{M_{sys}}{1+\left(q\frac{1+DK_1}{\tau_{sys}+(1+DK_b)}\right)^{m}} \quad 29$$

For the condition of no constitutive activity, equation 28 reduces to:

$$response = \frac{M_{sys}}{1+\left(\frac{1+DK_1}{qD\tau K_1}\right)^{m}} \quad 30$$

This equation can be rearranged into the following form:

$$response = \frac{M_{sys}}{1+\left(\frac{1+DK_1}{qD*R}\right)^m} \quad 31$$

in which R denotes the product, τK_1, as described above. For the case of comparing the τK_1 value of one agonist, relative to that of a standard agonist ($\tau' K_1'$), the following equation is useful:

$$response = \frac{M_{sys}}{1+\left(\frac{1+DK_1}{D*R*RA_i}\right)^m} \quad 32$$

in which, RA_i is defined as:

$$RA_i = \frac{\tau K_1}{\tau' K_1'} = \frac{\varepsilon K_1}{\varepsilon' K_1'} \quad 33$$

In this equation, the parameters designated with a prime symbol denote those of the standard agonist. It can be shown that RA_i is also a relative measure of the microscopic affinity constant of an agonist for the active state of a receptor, expressed relative to that of a standard agonist, as described previously (Tran, et al., 2009) and by equation 15 and the equivalency of τK_1 and K_{bqs}.

Equations 31 and 32 can also be used to estimate the RA_i values of both orthosteric and allosteric ligands when the latter are tested in the absence of other ligands (F.J. Ehlert, 2008; F. J. Ehlert, Suga, & Griffin, 2011b; Figueroa, Griffin, & Ehlert, 2008; Griffin, Figueroa, Liller, & Ehlert, 2007). They are also valid for the case of reduced receptor expression with no constitutive activity.

2.3 Simulation of agonist concentration-response curves

The simulation of theoretical concentration-response curves involved three steps: 1) generating a receptor activation function using a modification of the quaternary complex scheme, 2) substituting this function into a transducer function to generate theoretical responses, and 3) adding a random error.

The quaternary complex scheme (F.J. Ehlert, 2008; F. J. Ehlert & Rathbun, 1990), defined at the level of receptor states (active, R_s* and inactive, R_s), was used to simulate activation of the

receptor population. This scheme describes the interactions among agonist (*D*), receptor (*R*), G protein (*G*) and guanine nucleotide (*X*). We expanded our scheme to include an allosteric ligand for the receptor (*A*) and active and inactive states of the G protein (G^*_s and G_s, respectively). It was assumed that R_s^* exhibits high affinity for G_s^* and that GDP (*X*) exhibits high affinity for G_s. These conditions are consistent with the crystal structures of $G_{\alpha i}G_{\beta\gamma}$ bound with GDP (Lambright, et al., 1996; Wall, et al., 1995) and that of the β_2-adrenoceptor bound with agonist and $G_{\alpha s}G_{\beta\gamma}$ (Rasmussen, et al., 2011).

A simple form of the quaternary complex scheme for allosterism is shown in Figure 3*a* where only the sum of the active and inactive states of receptor (*R*) and *G* protein (*G*) are illustrated (i.e., $R = R_s + R_s^*$ and $G = G_s + G_s^*$). The scheme was expanded to account for the four possible combinations of states of the receptor and G protein (Figure 3*b*). We solved this scheme numerically to predict the amount of receptor-G protein complex in the active state bound with guanine nucleotide in the presence of various concentrations of allosteric and orthosteric ligand and guanine nucleotide. It was assumed that the eight possible combinations of G_s^*X bound with receptor and ligands could support guanine nucleotide exchange. The summation of these receptor complexes is designated as the total stimulus (*T*). Using reasonable parameter estimates, however, only the $DR_s^*G_s^*X$, $R_s^*AG_s^*X$, $DR_s^*AG_s^*X$ (ligand-activated) and $R_s^*G_s^*X$ (constitutively active) complexes accumulate in appreciable amounts. The method for solving this scheme is described under "Appendix".

To convert the total stimulus into a response, the reverse engineering approach described by Black and Leff (1983) was used (equation 2). Using a given set of parameter values, we solved the quaternary complex scheme for allosterism (equation 37 in the Appendix) numerically and substituted the result for *T* in equation 2 to simulate the response. These calculations were repeated for various concentrations of allosteric and orthosteric ligands.

A random error was added to the theoretical response values to simulate experimental variation. First, a constant value equivalent to 0.2 of M_{sys} was added to all of the simulated data because many responses exhibit a background measurement (e.g., basal fluorescence, cAMP or

[^{3}H]inositolphosphates) in the absence of receptor expression. A random error having a range of 40% of the measurement plus background (± 20%) was added. Then the background value was subtracted to avoid confusion between it and the constitutive stimulus. The net result is that the response values have greater than a 48% random error (i.e., ± 24%). For each analysis, we simulated four replicates, and the figures illustrate the mean ± SEM of these replicates.

2.4 Nonlinear regression analysis

Both simulated and experimental data were analyzed by global nonlinear regression analysis using Prism (GraphPad Software, San Diego, CA) or Excel (Microsoft, Mountain View, CA) and selected equations described above. For this analysis, the regression equations were rearranged so that the microscopic constants (K_a, K_b, K_e, K_f and K_q) and some of the population parameters (i.e., K_1, K_2, α, γ_1, τ, τ_A and τ_{sys}) were expressed as logarithms (e.g., $K_a = 10^{\log Ka}$). The estimates of these log parameters are given in the text ± their asymptotic standard error. For the analysis of the independent effects of the ligands (Figure 8), each replicate set of simulated data was analyzed, and the mean ± SEM of each parameter estimate is reported.

As shown in the Appendix, it is possible to express the population and microscopic constants in terms of the graphical parameters (EC_{50}, E_{max}, etc.) of the concentration-response curves for the case of $m = 1$. These equations can be used for calculating initial parameter estimates for nonlinear regression analysis regardless of whether $m = 1$.

In some cases it is impossible to estimate some of the microscopic and macroscopic (population) parameters in a given scheme. In these instances, we searched parameter space by constraining either K_a (receptor state analysis) or K_1 (population analysis) to a range of values and estimated the other parameters that minimized the residual sum of squares (*RSS*). Only those parameters whose estimates were constant over the domain that yielded a least-squares fit are reported.

3. Results

3.1 Quaternary complex scheme for allosterism

We used equation 2 and the method described in the Appendix to generate receptor activation functions. Examples of these are shown in Figure 4 for the case of a positive ($K_f/K_e = 10$) allosteric modulator and a highly efficacious ($K_b/K_a = 10^4$) agonist. The value of the isomerization constant (K_q) was 10^{-4}, and the concentration of guanine nucleotide (X) was 10^{-3} M. For the cases shown in Figure 4, fractional constitutive receptor activation (ε_{sys}) is barely detectable, but it can be enhanced by increasing the isomerization constant of the unoccupied receptor (K_q).

Each of these receptor-activation functions can be analyzed using a one-site equation to estimate the observed affinity constant of the orthosteric ligand (K_1). This was done for the simulated condition of no modulator using the following equation:

$$T = \frac{\varepsilon_{sys} + D\varepsilon K_1}{1 + DK_1} \qquad 34$$

in which, ε, denotes the efficacy of the orthosteric agonist, and ε_{sys}, the constitutive stimulus. In all cases, the Hill slopes were equivalent to one as determined by regression analysis with a logistic equation. The values of log K_1 and ε for the agonist were (4.16) and (0.21), respectively.

Maximal receptor activation approaches a value of 1.0 for the two-state scheme (Figure 2*a*, equation 2) at high concentrations of an agonist with a sufficiently high ratio of K_b/K_a. In contrast, the maximum of the quaternary complex scheme for allosterism is often less than one and depends on the ratio of G protein to receptor, the concentration of guanine nucleotide, and the various parameters in the model. For the simulations in Figure 4, maximum receptor activation with an agonist having infinite selectivity for the active state ($K_b/K_a = \infty$) is 0.68.

Although the maximum of the quaternary complex scheme for allosterism is less than 1.0, the regression equations developed to analyze downstream responses are based on a simple two-state scheme having a maximum of 1.0. This discrepancy gives rise to a difference between the K_E

value used to simulate downstream responses and the observed estimate of K_E ($K_{E\text{-}obs}$) determined by regression analysis of the simulated data. The relationship between K_E and $K_{E\text{-}obs}$ is given by:

$$K_{E-obs} = \frac{K_E}{T_{max}} \qquad 35$$

in which T_{max} represents the fractional maximum of the quaternary complex function for an agonist having infinite efficacy (0.68 for the example in Figure 4). The theoretical $K_{E\text{-}obs}$ values for the simulations described below were calculated using equation 35.

There is also a discrepancy between the K_q value used to simulate the receptor activation function and the $K_{q\text{-}obs}$ parameter of the simple scheme shown in Figure 2*a*. The latter theoretical value can be calculated numerically or estimated by fitting the receptor activation function (equation 1) to the control curve in Figure 4 with K_b and K_a constrained to their theoretical values and the maximum to 0.68. Using the latter approach, the theoretical value of log $K_{q\text{-}obs}$ was determined (-4.35). This observed value is reduced from that used to simulate the data (log K_q, -4.0) because of the influence of G protein and guanine nucleotide.

3.2 Analysis of simulated allosteric interactions, two-state scheme

We simulated agonist concentration-response curves for three conditions: 1) control, 2) after reduced receptor expression or after partial receptor inactivation with a ligand that behaves as a neutral antagonist when bound irreversibly (irreversible neutral antagonist), and 3) in the presence of various concentrations of allosteric ligand after reduced receptor expression or partial receptor inactivation. All of the parameters used to simulate data are given in the corresponding figure legends.

Simulated data for a positive allosteric modulator are shown in Figures 5*a* and *b* for conditions of reduced receptor expression and partial receptor inactivation, respectively. If there is no constitutive activity and the allosteric ligand lacks an effect by itself, then it is impossible to estimate K_b and $K_{q\text{-}obs}$, but it is possible to estimate K_{bqs}. In addition, there is little difference

between the effects of reduced receptor expression or partial receptor inactivation in this situation.

The data in Figure 5*a* were analyzed by global nonlinear regression analysis using equation 8, which yielded the following parameter estimates: q, 0.059 ± 0.016; log K_a, 4.02 ± 0.09; log K_{bqs}, 5.48 ± 0.04; log K_e, 5.11 ± 0.08; log K_f, 6.54 ± 0.08; log $K_{E\text{-}obs}$, -1.65 ± 0.16; M_{sys}, 1.01 ± 0.02 and m, 1.75 ± 0.24. Similarly, the data in Figure 5*b* were also analyzed using equation 8, and the following parameter estimates were obtained: q, 0.059 ± 0.016; log K_a, 4.12 ± 0.09; log K_{bqs}, 5.52 ± 0.04; log K_e, 5.00 ± 0.08; log K_f, 6.46 ± 0.08; log $K_{E\text{-}obs}$, -1.92 ± 0.15; M_{sys}, 0.95 ± 0.02 and m, 1.73 ± 0.24. All of these estimates are nearly the same as those used to simulate the data: q, 0.05; log K_a, 4.0; log K_{bqs}, 5.48; log K_e, 5.0; log K_f, 6.5; log $K_{E\text{-}obs}$, -1.83; M_{sys}, 1.0 and m, 1.5.

We also simulated data for an allosteric agonist for conditions of reduced expression (Figures 6*a* and *b*) and partial receptor inactivation (Figure 6*c*). The data for reduced expression are shown in two panels for clarity – panel *a* shows the control curves for the orthosteric and allosteric agonists and panel *b* shows their interaction under conditions of reduced receptor expression.

The data in Figure 6*a* and *b* were analyzed simultaneously by global nonlinear regression analysis using equation 11. This analysis yielded the following parameter estimates: q, 0.063 ± 0.013; log K_a, 4.14 ± 0.08; log K_b, 7.93 ± 0.11; log K_e, 5.02 ± 0.05; log K_f, 7.17 ± 0.07; log $K_{q\text{-}obs}$, -4.09 ± 0.22; log $K_{E\text{-}obs}$, -1.69 ± 0.13; M_{sys}, 0.96 ± 0.02 and m, 1.80 ± 0.13. Similarly, the data in Figure 6*c* were analyzed (equation 7), and the following parameter estimates were obtained: q, 0.056 ± 0.011; log K_a, 3.97 ± 0.08; log K_b, 7.90 ± 0.15; log K_e, 5.08 ± 0.09; log K_f, 7.19 ± 0.07; log $K_{q\text{-}obs}$, -4.07 ± 0.26; log $K_{E\text{-}obs}$, -1.64 ± 0.12; M_{sys}, 1.05 ± 0.02 and m, 1.63 ± 0.17. All of these estimates are nearly the same as those used to simulate the data: q, 0.05; log K_a, 4.0; log K_b, 7.0; log K_e, 5.0; log K_f, 7.2; log $K_{q\text{-}obs}$, -4.35; log $K_{E\text{-}obs}$, -1.83; M_{sys}, 1.0 and m, 1.5.

Finally, we simulated data for a negative allosteric modulator at a receptor exhibiting constitutive activity under conditions of reduced receptor expression (Figures 7*a* and *b*) and partial receptor inactivation (Figure 7*c*). The control concentration-response curves for the

orthosteric and allosteric ligands (panel *a*) and those of the agonist measured in the presence of various concentrations of the modulator after reduced receptor expression (panel *b*) were analyzed by global nonlinear regression analysis using equation 11, and the following parameter estimates were obtained: q, 0.013 ± 0.004; log K_a, 4.09 ± 0.08; log K_b, 6.95 ± 0.11; log K_e, 6.02 ± 0.06; log K_f, 5.07 ± 0.06; log $K_{q\text{-}obs}$, -2.27 ± 0.20; log $K_{E\text{-}obs}$, -2.13 ± 0.17; M_{sys}, 1.03 ± 0.02 and m, 1.63 ± 0.20. Similarly, the data in Figure 7*c* were analyzed using equation 7, and the following parameter estimates were obtained: q, 0.019 ± 0.004; log K_a, 3.93 ± 0.08; log K_b, 6.88 ± 0.14; log K_e, 6.19 ± 0.12; log K_f, 4.84 ± 0.12; log $K_{q\text{-}obs}$, -2.35 ± 0.18; log $K_{E\text{-}obs}$, -2.11 ± 0.15; M_{sys}, 1.07 ± 0.02 and m, 1.07 ± 0.18. All of these estimates are nearly the same as those used to simulate the data: q, 0.01; log K_a, 4.0; log K_b, 7.0; log K_e, 6.0; log K_f, 5.0; log $K_{q\text{-}obs}$, -2.41; log $K_{E\text{-}obs}$, -2.26; M_{sys}, 1.0 and m, 1.5.

3.3 Analysis of simulated allosteric interactions, population scheme

In many instances, the nature of allosteric modulation of receptor function is inconsistent with a two-state scheme. Nonetheless, allosterism can always be analyzed at the more superficial level of the average behavior of the receptor population (ensemble average). In this section, we summarize the analysis of the simulated data in Figures 5 – 7 using the population scheme (Figure 2*b*, equations 25 and 26).

As described in the Appendix, each population parameter of the receptor activation function (τ_{sys}, K_1, τ, K_2, τ_A, α, β and γ) can be expressed as a function of microscopic constants (see equations 55 – 61, 63 – 66, 68). The theoretical population parameters were calculated from the microscopic constants used to simulate the data, and these are given below.

The data in Figure 5*a* were reanalyzed using the population model for reduced receptor expression (equation 26) with the estimates of τ_{sys} and τ_A constrained to zero (i.e., log τ_{sys} = -20 and log τ_A = -20) because this simulation represents data for a receptor lacking constitutive activity and a modulator lacking an effect by itself. Global nonlinear regression analysis of the data yielded the following parameter estimates: q, 0.049 ± 0.009; log K_1, 4.17 ± 0.08; log τ, 1.33

± 0.08; log K_2, 5.07 ± 0.06; log γ_1, 1.50 ± 0.07; log α, 1.08 ± 0.08; M_{sys}, 1.01 ± 0.02 and m, 1.57 ± 0.13. Similarly, global nonlinear regression analysis of the data in Figure 5*b* was analyzed by the population model for partial receptor inactivation (equation 25) and the following parameter estimates were obtained: q, 0.044 ± 0.008; log K_1, 4.17 ± 0.08; log τ, 1.38 ± 0.09; log K_2, 4.92 ± 0.07; log γ_1, 1.59 ± 0.08; log α, 1.01 ± 0.10; M_{sys}, 0.96 ± 0.02 and m, 1.47 ± 0.12. All of these estimates are nearly the same as those used in the simulation: q, 0.05; log K_1, 4.16; log τ, 1.32; log K_2, 5.00; log γ_1, 1.5; log α; 1.02; M_{sys}, 1.0 and m, 1.5.

We also analyzed the simulation for an allosteric agonist using the population model (Figure 6). Global nonlinear regression analysis of the data in Figure 6*a* and *b* using equation 26 with τ_{sys} constrained to zero (log τ_{sys} = -20) yielded the following parameter estimates: q, 0.062 ± 0.013; log K_1, 4.31 ± 0.09; log τ, 1.22 ± 0.09; log K_2, 5.02 ± 0.06; log τ_A, -0.26 ± 0.05; log γ_1, 2.15 ± 0.09; log α, 1.67 ± 0.09; M_{sys}, 0.96 ± 0.02 and m, 1.80 ± 0.17. Similarly, global nonlinear regression analysis of the data in Figure 6*c* using the population model for partial receptor inactivation (equation 25) yielded the following parameter estimates: q, 0.056 ± 0.011; log K_1, 4.21 ± 0.09; log τ, 1.26 ± 0.09; log K_2, 5.08 ± 0.10; log τ_A, -0.32 ± 0.04; log γ_1, 2.11 ± 0.12; log α, 1.73 ± 0.11; M_{sys}, 1.05 ± 0.02 and m, 1.62 ± 0.16. All of these estimates are nearly the same as those used in the simulation: q, 0.05; log K_1, 4.16; log τ, 1.32; log K_2, 5.0; log τ_A, -0.33; log γ_1, 2.20; log α, 1.69; M_{sys}, 1.0 and m, 1.50.

Finally, we reanalyzed the data in Figure 7 using the population model. Global nonlinear regression analysis of the simulated data in Figure 7*a* and *b* with the population model for reduced receptor expression (equation 26) yielded the following parameter estimates: q, 0.017 ± 0.007; log τ_{sys}, -0.13 ± 0.08; log K_1, 4.85 ± 0.11; log τ, 1.91 ± 0.20; log K_2, 6.03 ± 0.06; log τ_A, -0.87 ± 0.19; log γ_1, -0.94 ± 0.08; log α, -0.56 ± 0.07; M_{sys}, 1.02 ± 0.01 and m, 1.79 ± 0.29. Similarly, global nonlinear regression analysis of the data in Figure 7*c* using the population model for partial receptor inactivation (equation 25) yielded the following parameter estimates: q, 0.019 ± 0.004; log τ_{sys}, -0.23 ± 0.05; log K_1, 4.61 ± 0.09; log τ, 2.01 ± 0.17; log K_2, 6.17 ± 0.12; log τ_A, -1.59 ± 0.44; log γ_1, -1.32 ± 0.21; log α, -0.61 ± 0.10; M_{sys}, 1.08 ± 0.02 and m, 1.08

± 0.22. All of these estimates are nearly the same as those used in the simulation: q, 0.01; log τ_{sys}, -0.15; log K_1, 4.69; log τ, 2.16; log K_2, 6.0; log τ_A, -1.15; log γ_1, -1.0; log α, -0.55; M_{sys}, 1.0 and m, 1.50.

Knowing the sensitivity constant of the signaling pathway ($K_{E\text{-}obs}$), the τ values from the population analysis (i.e., τ, τ_A and τ_{sys}) and the microscopic constants, it is possible to estimate the efficacy of the ligands and the constitutive activity of the signaling pathway using equations 18 – 20 and 57 – 59 (Appendix). These calculations yield efficacy estimates for the orthosteric (ε) and allosteric (ε_A) ligands and the free receptor (constitutive activity, ε_{sys}) for the data from Figure 5*b* (ε, 0.29) 6*c* (ε, 0.42; ε_A, 0.011; ε_{sys}, 8.1×10^{-5}) and 7*c* (ε, 0.80; ε_A, 2.0 x 10^{-4}; ε_{sys}, 4.5×10^{-3}). Note that in the last case, which involves an allosteric inverse agonist, efficacy (ε_A) is less than constitutive activity (ε_{sys}). The same would be true for the case of an orthosteric inverse agonist (F. J. Ehlert, Suga, et al., 2011a).

3.4 Analysis of the independent activity of ligands

The nature of the interaction between orthosteric and allosteric ligands depends on their selectivity for active and inactive states and the isomerization constant of the receptor. Thus, the microscopic constants estimated for an allosteric interaction between a pair of orthosteric and allosteric ligands should be the same as those estimated from their independent effects provided that the same active state of the receptor mediates the response in both situations. In this section, we address this issue.

The independent effects of some of the ligands used in the simulations are plotted again in Figure 8 for convenience. The data include the orthosteric and allosteric agonists from Figure 6*a* (panel *a*); and Figure 6*c* (panel *b*) and the orthosteric agonist and negative allosteric modulator from Figure 7*a* (panel *c*) and Figure 7*c* (panel *d*).

The data in Figure 8*a* were analyzed by global nonlinear regression analysis using equations 13 and 14 as described under Methods. Regression analysis yielded estimates of q (0.047 ± .010), M_{sys} (0.98 ± 0.02), m (1.49 ± 0.14), log K_{bqs}, (5.50 ± 0.03) and the log K_a and log RA_i

values of the allosteric agonist (4.79 ± 0.08 and -1.02 ± 0.05). Similarly, regression analysis of the simulated data in Figure 8*b* yielded the following parameter estimates: q (0.06 ± .02), M_{sys} (1.04 ± 0.02), m (2.04 ± 0.35), log K_{bqs} (5.46 ± 0.03) and the log K_a and log RA_i values of the allosteric agonist (5.13 ± 0.14 and -0.60 ± 0.20). All of these are reasonable estimates of the theoretical values used to simulate the data for the interactive effects of the ligands shown in Figure 6: q, 0.05; M_{sys}, 1.0; m, 1.5; log K_{bqs} 5.48, log RA_i (log K_f/K_b), -1.0 and the log K_a, of the allosteric ligand (log K_e) 5.0.

The data in Figure 8*c* were analyzed using equation 9. Regression analysis was done sharing all of the parameters except K_a, K_b and q. The K_a and K_b values of the orthosteric agonist were shared under control and receptor inactivation conditions, whereas unique values were estimated for the negative allosteric modulator. The parameter q was estimated for the condition of receptor inactivation and constrained to 1.0 otherwise. Regression analysis yielded estimates of M_{sys}, (1.00 ± 0.02), m (1.46 ± 0.16), q (0.010 ± 0.002), the log K_b value of the orthosteric agonist (7.11 ± 0.05) and the log K_b and log K_a values of the negative allosteric modulator (4.77 ± 0.28 and 5.94 ± 0.091). The data in Figure 8*d* were analyzed in an analogous manner but with equation 12. Global nonlinear regression analysis yielded estimates of M_{sys}, (1.09 ± 0.05), m (1.44 ± 0.66), q (0.024 ± 0.004), the log K_b value of the orthosteric agonist (7.03 ± 0.34) and the log K_b and log K_a values of the negative allosteric modulator (4.35 ± 0.66 and 5.83 ± 0.14).

With the exception of the K_b value of the allosteric ligand, the latter values are nearly the same as those used to simulate the data for the interactive effects of the ligands illustrated in Figure 7: q, 0.01; M_{sys}, 1.0; m, 1.5; log K_b value of the orthosteric agonist, 7.0; and the log K_b and K_a values of the allosteric ligand (log K_f and K_e), 5.0 and 6.0, respectively. An accurate value for the K_b of a negative allosteric modulator is difficult to estimate whenever the responses measured in the presences of maximally effective concentrations of the modulator are negligible. This rationale explains the error in this parameter noted above.

3.4 Analysis of data from the literature

We analyzed two examples of data from the literature involving allosteric modulation of M_1 and M_2 muscarinic receptor function to determine if the two-state scheme shown in Figure 2 adequately described the allosteric effects.

The data in Figure 9*a* were estimated from a published figure by Canals and coworkers (2012). These investigators studied the effects of various concentrations of BQCA (1-(4-methoxybenzyl)-4-oxo-1,4-dihydro-3-quinoline carboxylic acid) on the concentration-response curves of carbachol for eliciting activation of a chimeric G protein containing the carboxyl terminus of $G\alpha_{i1,2}$ in a yeast cell expressing the human M_1 muscarinic receptor. We analyzed the data using receptor state equation 3 with all of the parameters shared during global nonlinear regression analysis. This analysis yielded estimates of log K_b (8.05 ± 0.13), log K_e (4.49 ± 0.09), log K_f (6.49 ± 0.09), M_{sys} (103 ± 1.5%) and m (0.85 ± 0.06). Because the interaction was not measured with reduced receptor expression or partial receptor inactivation, it was impossible to estimate K_a, $K_{q\text{-}obs}$ and $K_{E\text{-}obs}$. By searching parameter space, it was possible to estimate the domain of log K_a (log $K_a \leq 4.4$), however. In addition, regression analysis with equation 5 yielded estimates of log K_{bqs} (5.76 ± 0.06), and hence, log $K_{q\text{-}obs}/K_{E\text{-}obs}$ (-2.28 ± 0.15).

The second example is from our prior work on the allosteric effect of gallamine on oxotremorine-M-mediated inhibition of forskolin-stimulated cAMP accumulation in CHO cells expressing the human M_2 muscarinic receptor (Figure 9*b*) (F. J. Ehlert & Griffin, 2008). Concentration-response curves for oxotremorine-M were measured under control conditions and after partial inactivation of the receptor population with 4-DAMP mustard (N-(2-chloroethyl)-4-piperidinyl diphenylacetate). Under the latter condition, responses were measured in the absence and presence of gallamine (0.01 and 0.1 mM). The data were analyzed by a modified form of equation 7 to account for an inhibitory response, receptor inactivation, and no constitutive activity:

$$response = P_1 - \frac{P_1 M_{sys}}{1 + K_{E-obs}^{m}\left(\frac{1}{q} + \frac{(DK_a+1)(AK_e+1)}{qK_{q-obs}(DK_b+1)(AK_f+1)}\right)^{m}} \qquad 36$$

In this equation, P_1 denotes the amount of cAMP accumulation elicited by forskolin in the absence of other drugs, and in this case, M_{sys} denotes the maximal fractional inhibition of cAMP accumulation elicited by an agonist with infinity efficacy. Regression analysis yielded estimates of the microscopic constants log K_b (8.21 ± 1.08), log K_e (5.65 ± 0.18) and log K_q (-2.60 ± 1.12) as well as the transducer parameters of log $K_{E\text{-}obs}$ (-1.00 ± 0.26), M_{sys} (0.62 ± 0.09), m (1.042 ± 0.29) and P_1 (101± 2.21). The estimates of the microscopic affinity constants of the orthosteric and allosteric ligand for the inactive state of the receptor (K_a and K_e, respectively) were essentially equivalent to zero (large negative log values), however. These microscopic constants yielded reasonable estimates of the observed affinity constants for the orthosteric (log $K_1 = 5.61$) and allosteric (log $K_2 = 5.65$) ligands. The calculated estimate of log γ_1 was a large negative number, corresponding essentially to $\gamma_1 = 0$. Prior estimates of log γ_1 are in the range of -2, however. Thus, the two-state scheme does not provide a satisfactory fit to the data, when compared to the results of other studies where higher concentrations of gallamine were investigated.

We also attempted to fit the regression equation to the data with the log K_a value for oxotremorine-M constrained to a range of values estimated in prior studies on M_2 receptor-mediated inhibition of cAMP accumulation in HEK 293 cells (log $K_a = 4 – 5$). It was impossible to obtain a good fit to the data with this constraint, and the calculated value for the log observed affinity constant of gallamine (log K_2, 2.4 – 5.1) was much lower than that estimated in prior functional studies (~ 6.0).

When the population scheme was fitted to the data, a good fit was obtained. The estimates of log γ_1 and log α were unreliable because of the limited range of concentrations of gallamine. Constraining log γ_1 to values (1.8 – 2.3) estimated in prior studies (F. J. Ehlert, 1988b; F. J. Ehlert & Griffin, 2008) did not worsen the fit and yielded estimates of log α essentially equivalent to log γ_1. Thus, from the population perspective, gallamine appears to reduce the affinity of oxotremorine-M while having little effect on its efficacy.

4. Discussion

As described previously (F. J. Ehlert, 2000; F. J. Ehlert & Rathbun, 1990; Tran, et al., 2009), the quaternary complex scheme is useful for simulating receptor activation at a GPCR. It is based on the assumption that the signal elicited by the agonist-receptor complex is proportional to the fraction of the complex in the active state associated with GDP-bound G protein. This species catalyzes guanine nucleotide exchange and is, therefore, proportional to the activating stimulus that elicits downstream responses.

This scheme was solved for equilibrium conditions, yet during receptor signaling, the G protein achieves steady state. The factor that drives the system away from equilibrium is the GTPase activity of the G protein, which converts GTP to GDP. But because both of these guanine nucleotides have the same effect on agonist binding to many GPCRs (Berrie, Birdsall, Burgen, & Hulme, 1979; Childers & Snyder, 1978; Freedman, Poat, & Woodruff, 1981), the agonist-receptor complex should be at equilibrium, which justifies the use of the model for the purposes described in this report.

In this study, we have added allosteric ligands and active and inactive states of the G protein to our scheme. The latter modification allows the modeling of two counterintuitive phenomena: 1) guanine nucleotide-insensitive agonist binding and 2) little change in the amount of G protein associated with receptor upon agonist activation. The former can occur when the equilibrium between active and inactive states of the free G protein is shifted far to the inactive state and the latter whenever the inactive GDP-bound form of the holo G protein is precoupled to the receptor. These two phenomena can explain 1) the lack of prominent GTP effects on agonist affinity for G_q-coupled receptors (Katz & Miledi, 1970) and 2) some unexpected changes in agonist-mediated resonance energy transfer between receptors and G proteins (Derksen, 1965; Lindemann & DeFelice, 1981). As described previously, our scheme also adequately simulates GTP-sensitive agonist binding and agonist-induced formation of the receptor-G protein complex (F. J. Ehlert, 2000; F. J. Ehlert & Rathbun, 1990; Tran, et al., 2009).

We have shown through simulation that it is possible to estimate microscopic constants under specific conditions. This approach was taken for two reasons – to describe our method of analysis and to prove that microscopic constants can be estimated from functional data. The latter goal can be achieved more directly with an analytical proof. In the Appendix, we show that it is possible to calculate the population parameters and microscopic constants from the graphical parameters of the concentration-response curves for the condition where the transducer slope factor (m) in the operational model (equation 2) is equal to one. We have derived equations for the graphical parameters (see Figure 10) in terms of the population parameters of the allosteric ternary complex model (see Figure 2*b*). Then we solve these equations to express the population parameters in terms of the graphical parameters. Finally, using the relationships between microscopic and population parameters, the microscopic constants can be estimated from the population parameters. We verified the equations by using them to analyze theoretical data without error like those shown in Figure 10.

These equations illustrate what can be estimated from a given set of data. For example, it is difficult to obtain a reliable estimate of M_{sys} using a partial agonist and the method of partial receptor inactivation. The estimation of M_{sys} requires an accurate calculation of the difference between the EC_{50} values of the agonist measured before and after receptor inactivation (i.e., EC_1 – EC_2; see equations 96 and 148). This difference is difficult to estimate accurately because treatment of the receptor population with an irreversible antagonist mainly reduces the E_{max} of a partial agonist while having little effect on EC_{50}. The solution is to analyze the data simultaneously with a full agonist.

We also derived equations for expressing K_{bqs} and K_a in terms of the graphical parameters of the concentration-response curves when these are estimated independently from data like that illustrated in Figure 8, for example, for the condition of $m = 1$ (see Appendix). We previously described analogous equations for expressing relative (RA_i) and absolute estimates of K_b in terms of the graphical parameters of the concentration-responses curves of orthosteric agonists (F. J. Ehlert, Suga, et al., 2011a; Griffin, et al., 2007). All of these equations can be used to calculate

the microscopic affinity constants of allosteric agonists and inverse agonists when the concentration-response curves are measured independently. If there is a difference between parameters estimated from the independent and interactive effects of the orthosteric and allosteric ligands, then it is likely that the interacting ligands select for different active states of the receptor.

Although the equations derived under "Appendix" only apply to the condition of $m = 1$, second messenger responses often exhibit concentration-response curves with Hill slopes of one ($n = 1$, and hence, $m = 1$). Thus, these equations are useful in many cases, not withstanding the more robust approach of using the nonlinear regression methods that we describe in this report. More importantly, these equations can be used to calculate initial parameter estimates for the nonlinear regression analyses when the transducer slope factor, m, differs from 1.0. This would enable the development of a computer program for the analysis that requires no input from the investigator other than the raw data and the appropriate regression equation.

The minimum data necessary for estimating all of the population and microscopic parameters include the concentration-response curves of the orthosteric ligand measured under control conditions and after reduced receptor expression or partial receptor inactivation in the absence and presence of various concentrations of allosteric ligand. For the case no constitutive activity, an allosteric agonist is required for estimation of microscopic constants, but not population parameters. If used in conjunction with reduced receptor expression, the concentration-response of the allosteric agonist under control conditions is also required for estimation of microscopic constants for the case of no constitutive activity. The concentration of allosteric ligand should vary from a low value causing only about a two-fold shift in the concentration-response curve of the orthosteric ligand to a high value that saturates the allosteric site and elicits a near maximal effect. These conditions are likely to reveal an allosteric change in the E_{max} of the orthosteric ligand, which is essential for estimating $K_{q\text{-}obs}$ and K_E. If none is seen, then the ligand might not conform to a two-state scheme, and in such a case, only the population parameters can be determined.

For example, gallamine causes a large allosteric reduction in the observed affinity of some agonists for the M_2 muscarinic receptor without affecting efficacy (F. J. Ehlert, 1988b; F. J. Ehlert & Griffin, 2008). This mechanism is clearly inconsistent with a two-state scheme as described under "Results" in connection with Figure 9*b*. The mechanism is also difficult to rationalize with a multi-state scheme, and we have suggested that perhaps gallamine regulates a relay site on the muscarinic receptor and not the orthosteric binding pocket (F. J. Ehlert & Griffin, 2008).

Hulme (2003) has suggested that a residue in helix four in the human M_1 sequence (W157; W155 in M_2) might function as a docking site where acetylcholine first binds before shuttling to the orthosteric site. In a recent modeling study based on the crystal structure of the M_2 muscarinic receptor, it was shown that the antagonist tiotropium interacts with W155 on the M_2 receptor when traversing to and from the orthosteric site (Kruse, et al., 2012). Perhaps this relay process undergoes allosteric modulation by gallamine. Because the population scheme is unconstrained by microscopic constants, it yields appropriate parameter estimates for a relay model. In this case, the estimate of the population affinity constant (K_{obs}) is actually a weighted composite of the affinities of the relay and orthosteric sites (F. J. Ehlert & Griffin, 2008).

It is often assumed that the value of the observed affinity constant (K_{obs}) of an agonist for a GPCR in its native context is nearly the same as that of its microscopic affinity constant for the inactive state because high intracellular concentrations of GTP greatly shift the equilibrium between the active and inactive states in the direction of the inactive state (F. J. Ehlert, 2000; Strange, 2007). There is circumstantial evidence, however, that in some instances the value of K_{obs} is substantially greater than K_a. When estimated by the method of partial receptor inactivation, log K_{obs} values of 3.74 ± 0.14 and 4.00 ± 0.13 were estimated for carbachol and oxotremorine-M in experiments on human M_3 muscarinic receptor-mediated phosphoinositide hydrolysis in HEK293 cells (F. J. Ehlert, Suga, et al., 2011a). With regard to M_3 muscarinic receptor-mediated contraction in mouse ileum, the same method of analysis yielded much higher log K_{obs} values of 5.49 ± .14 and 5.73 ± .14 for carbachol and oxotremorine-M, respectively

(Tran, et al., 2009). In CHO cells stably transfected with the human M_2 receptor, analysis of carabachol- and oxotremorine-M-mediated inhibition of cAMP accumulation yielded log K_{obs} values of 5.36 ± .18 and 6.54 ± 0.14, respectively, yet the log binding affinities of these agonists in homogenates of the rat and rabbit myocardium are only about 4.0 and 5.4 when measured in the presence of GTP (0.1 mM) (F. J. Ehlert, 1985; F. J. Ehlert & Rathbun, 1990). Thus, in functioning receptosomes of the ileum and CHO cells, but not HEK 293 cells, the local concentration of GTP may be less than that required to saturate G proteins.

In cellular homogenates, of course, the concentration of GTP can be manipulated, and the estimate of K_{obs} can be much greater than that of K_a whenever the concentration of guanine nucleotides is less than saturating. Even under the latter condition, it is theoretically possible for K_{obs} to exceed K_a. This issue has relevance to common assays employing tissue and cellular homogenates, like adenylate cyclase and [^{35}S]GTPγS binding. Not surprisingly, when functional and binding assays were carried out at the same concentration of GTP, similar values of K_{obs} were estimated for a given agonist at the M_2 muscarinic receptor in the heart (F. J. Ehlert, 1987).

We previously described an approach for estimating the K_a values of a series of agonists through analysis of their concentration-response curves independently of allosteric modulation (F. J. Ehlert, Griffin, et al., 2011). The analysis involved first constraining the sensitivity constant ($K_{E\text{-}obs}$) in the operational model to the largest value that yielded a least-squares fit for the most efficacious agonist in a series (i.e., estimate of $K_{E\text{-}obs}$ when $\varepsilon = 1.0$) and then using a regression scheme to estimate the K_a values of less efficacious agonist. This approach yields reasonable K_a values for agonists provided that their efficacies are less than one-half that of the most efficacious agonist. The method described in this report employing regression equations 13 and 14 (see Figure 8) is an improvement and also yields estimates of the standard error of log K_a.

The same analysis also yields an estimate of the RA_i value of an agonist, which represents the product of the affinity and efficacy of the agonist expressed relative to that of another agonist (standard agonist). RA_i is also equivalent to the corresponding ratio of K_b values. The theoretical basis for this estimate was first described by Ehlert et al. (1999) using a response clamp analysis

(null method) as well as in subsequent publications where explicit methods on how to calculate this parameter are given (F.J. Ehlert, 2008; F. J. Ehlert, Suga, et al., 2011b; Griffin, et al., 2007). A similar method for estimating the same parameter has been recently described by Kenakin and coworkers (2012).

We have described our methods from the perspective of the orthosteric agonist. The analysis can also be done from the opposite perspective – that is, measuring the effect of an orthosteric ligand on the concentration-response curve of an allosteric agonist. The same equations are used, except that the concentration of allosteric ligand would represent the main independent variable. Measuring the effect of an orthosteric inverse agonist on the concentration-response curve of an allosteric agonist provides a means of estimating the affinity of the inverse agonist for the active state of the receptor, which is difficult to estimate in the absence of allosteric modulation if the inverse agonist has high selectivity for the inactive state (F. J. Ehlert, Suga, et al., 2011a).

Our method enables the estimation of the isomerization constant of the receptor ($K_{q\text{-}obs}$) and the parameters of the transducer function ($K_{E\text{-}obs}$, M_{sys} and m, equation 2) for signaling pathways in cell lines, primary cells and tissues. Knowing these constants, one could estimate the K_b and K_a values of agonists and inverse agonists (orthosteric or allosteric) by regression analysis of their concentration-response curves using a simplified form of equation 3 (for the condition $A = 0$) with $K_{q\text{-}obs}$ and the parameters of the transducer function constrained to their previously determined values. Alternatively, global nonlinear regression analysis of the concentration-response curves with the data for the allosteric modulation of a full agonist could be done.

The active state of a GPCR is an adaptation that enables the rapid transfer of information through its complementary signaling protein (e.g., G protein). It is the first cause of all downstream events. Thus, the affinity constant of an agonist for the active state is an ideal measure of agonist bias provided that its value is sufficiently greater than that of the inactive state. Arguing from the perspective of the receptor population, several authors conclude that G proteins determine the activity of agonists. While the abundance and type of G protein can

certainly modify observed affinity (K_1 or K_2) and efficacy (ε) of an agonist for a particular signaling pathway, it seems unlikely that the interaction has any effect on K_b and K_a. Rather, G proteins provide a window for monitoring the activity of different effector-selective states of the receptor (F. J. Ehlert, Suga, et al., 2011a). Our method provides a means of estimating the affinity of agonists for these states.

Studies on structure-activity relationships often involve an attempt to relate modification in agonist structure to a change in activity usually estimated as EC_{50} or observed binding affinity (K_1). For a highly efficacious agonist, however, there is no real receptor structure the has an affinity constant of K_1. Rather, there are at least two types of complexes (active and inactive), and our method provides the appropriate affinity constants for these (K_b and K_a). Hence, a more fundamental understanding of structure-activity relationships can be achieved using our analysis. It also provides a means to validate *in silico* computations of the affinity constants of a ligand for crystal structures of active and inactive receptor states.

One final point is that we have explained our method by considering the simple example of a receptor with one active and one inactive state. Our approach can be modified to account for more than one active state (see Tran et al. (2009) and Ehlert & Griffin (2008)). With sufficient data, including different responses and effector-selective agonists, it should be possible to resolve the microscopic constants of two or three active states.

5. Appendix

5.1 *Numerical solution to the quaternary complex scheme for allosterism*

The activation function for the quaternary complex scheme for allosterism (Figure 3*c*) represents the sum of the receptor complexes associated with the active state of the guanine nucleotide-occupied G protein divided by the sum of all of the receptor complexes (R_T):

$$T = \frac{DR_S^*G_S^*X + DR_S^*AG_S^*X + R_S^*AG_S^*X + R_S^*G_S^*X + DR_SG_S^*X + DR_SAG_S^*X + R_SAG_S^*X + R_SG_S^*X}{R_T} \qquad 37$$

Four sets of receptor complexes contribute to R_T. The first includes all receptor complexes in which the receptor and G protein, if the latter is associated with the receptor, are in the inactive state: DR_s, R_sA, DR_sA, R_s, DR_sG_s, R_sAG_s, DR_sAG_s, R_sG_s, DR_sG_sX, R_sAG_sX, DR_sAG_sX and R_sG_sX. The second includes the same complexes but with each protein in the active state. The third includes only those complexes having both the active state of the receptor and the inactive state of the G protein. The fourth set is analogous to the third except that the G protein is in the active state and the receptor is in the inactive state.

Each receptor complex can be expressed as a function of microscopic constants, the inactive state of the free receptor concentration, and various ligand concentrations. The microscopic constants are defined as:

$$K_a = \frac{[DR_S]}{[D][R_S]} \qquad 38 \qquad K_b = \frac{[DR_S^*]}{[D][R_S^*]} \qquad 39$$

$$K_e = \frac{[AR_S]}{[A][R_S]} \qquad 40 \qquad K_f = \frac{[AR_S^*]}{[A][R_S^*]} \qquad 41$$

$$K_g = \frac{[R_SG_S]}{[G_S][R_S]} \qquad 42 \qquad K_h = \frac{[R_S^*G_S]}{[R_S^*][G_S]} \qquad 43$$

$$K_j = \frac{[R_SG_S^*]}{[R_S][G_S^*]} \qquad 44 \qquad K_k = \frac{[R_S^*G_S^*]}{[R_S^*][G_S^*]} \qquad 45$$

$$K_l = \frac{[G_SX]}{[G_S][X]} \qquad 46 \qquad K_m = \frac{[G_S^*X]}{[G_S^*][X]} \qquad 47$$

$$K_q = \frac{[R_S^*]}{[R_S]} \qquad 48 \qquad K_r = \frac{[G_S^*]}{[G_S]} \qquad 49$$

Note that, at the level of receptor states, the affinity of a ligand is determined solely by the state of the protein to which it binds and is independent of whether other ligands or proteins are associated with its binding protein. For example, the microscopic affinity constant, K_a, defines all of the following equilibria:

$$K_a = \frac{[DR_S]}{[D][R_S]} = \frac{[DR_SA]}{[D][R_SA]} = \frac{[DR_SAG_S^*]}{[D][R_SAG_S^*]} = \frac{[DR_SAG_S^*X]}{[D][R_SAG_S^*X]} \qquad 50$$

Using the microscopic constants described above, it is possible to derive an equation for each receptor complex described in equation 37. For example:

$$[DR_s^*AG_s^*X] = [D][X][G_s]K_bK_kK_mK_qK_r[R_s] \quad 51$$

The resulting equations for all of the receptor complexes are substituted into equation 37. In our calculations, we substituted the following expression for the free concentration of G protein (G_s):

$$G_s = \frac{G_T}{R_T} * \frac{1}{1+K_r} \quad 52$$

In which the ratio, G_T/R_T, denotes the ratio of total G protein (G_T) to total receptor (R_T).

Thus, by using substitutions for each receptor complex, it is possible to solve equation 37 for a given set of values for the microscopic constants, concentrations of the various ligands (D, A and X), and ratio of G_T/R_T.

When the ratio, G_T/R_T, is not large, however, the free concentration of G protein (G_s) in the plasma membrane decreases with an increase in receptor occupancy by agonists. To correct for this reduction in G_s, we used the following iteration procedure to determine the relative amount of free G protein:

$$G_{i+1} = G_i + \left(\frac{G_T}{R_T} - (G_i + G_b)\right) \quad 53$$

In this equation, G_i and G_b denote the ratio of free and bound G protein to total receptor, respectively, for the current iteration, and G_{i+1} denotes the corresponding ratio for the subsequent iteration. G_b is calculated as the subset of R_T that includes the 16 possible receptor complexes that contain a state of R (R_s or R^*_s) bound to a state of G (G_s or G*s) divided by R_T. Using this iteration procedure, the value of G_i reached a constant value within about three iterations, and we routinely carried out the calculation for 12 iterations.

The total stimulus for the condition of partial receptor inactivation was calculated with the following equation:

$$T = T_{residual} + T_{inact} \quad 54$$

In this equation, T_{resid} and T_{inact} denote the stimulus functions for the residual and alkylated receptors, respectively. The function for T_{resid} is equivalent to the right side of equation 37 multiplied by q, which denotes the fraction of the receptor population not alkylated by the irreversible antagonist. T_{inact} is also equivalent to the right side of equation 37 after eliminating all of the variables for receptor complexes containing the orthosteric ligand (D) and multiplying the result by $1 - q$. For the condition of reduced receptor expression, the total stimulus is equal to $T_{residual}$.

5.2 *Relation between the population parameters and microscopic constants*

The relationships between most of the population parameters and the microscopic constants (equations 55 – 61, 63, 64,66) have been described previously (F. J. Ehlert & Griffin, 2008; F. J. Ehlert, Suga, et al., 2011a) and are listed below for convenience. The observed affinity constant of the orthosteric (K_1) and allosteric (K_2) ligands for their sites on the receptor is given by:

$$K_1 = \frac{K_a + K_b K_{q-obs}}{1 + K_{q-obs}} \quad 55$$

$$K_2 = \frac{K_e + K_f K_{q-obs}}{1 + K_{q-obs}} \quad 56$$

The equations for the parameters τ, τ_A and τ_{sys} are given by:

$$\tau = \frac{1}{K_{E-obs}\left(1 + \frac{K_a}{K_b K_{q-obs}}\right)} \quad 57$$

$$\tau_A = \frac{1}{K_{E-obs}\left(1 + \frac{K_e}{K_f K_{q-obs}}\right)} \quad 58$$

$$\tau_{sys} = \frac{K_{q-obs}}{K_{E-obs}(1 + K_{q-obs})} \quad 59$$

Using these functions for K_{obs} (i.e., K_1 or K_2), τ, τ_A and τ_{sys}, it can be shown that

$$K_b = \frac{\tau K_1}{\tau_{sys}} \quad 60$$

$$K_f = \frac{\tau K_2}{\tau_{sys}} \quad 61$$

The compound parameter, K_{bqs}, is equivalent to (see equations 4, 55 and 57):

$$K_{bqs} = K_1\tau \quad 62$$

The constant describing the reciprocal allosteric change in observed affinity (α) that each ligand has on the other is given by:

$$\alpha = \frac{(1+K_{q-obs})(K_aK_e+K_bK_fK_{q-obs})}{(K_e+K_fK_{q-obs})(K_a+K_bK_{q-obs})} \quad 63$$

The scalar change in the efficacy of the orthosteric ligand caused by the allosteric ligand (β_1) and that of the orthosteric ligand on the efficacy of the allosteric ligand (β_2) are given by:

$$\beta_1 = \frac{K_aK_f+K_bK_fK_{q-obs}}{K_aK_e+K_bK_fK_{q-obs}} \quad 64$$

$$\beta_2 = \frac{K_bK_e+K_bK_fK_{q-obs}}{K_aK_e+K_bK_fK_{q-obs}} \quad 65$$

The product of the scalar changes in the affinity (α) and efficacy (β_1) of the orthosteric ligand ($\alpha\beta_1 = \gamma_1$) induced by the allosteric ligand is given by:

$$\gamma_1 = \frac{K_f+K_fK_{q-obs}}{K_e+K_fK_{q-obs}} \quad 66$$

By making the appropriate substitutions for K_2 and γ_1 (equations 58 and 68, respectively), it can be shown that:

$$\gamma_1K_2 = K_f \quad 67$$

This rather simple relationship explains why γ_1 is one of the easiest parameters to estimate from functional data. The estimates of γ_1 for the various simulations are all in close agreement with the ratio, K_f/K_e. Finally, the scalar effect of the orthosteric ligand on the $\alpha\beta_2$ value of the allosteric ligand (γ_2) is given by:

$$\gamma_2 = \frac{K_b+K_bK_{q-obs}}{K_a+K_bK_{q-obs}} \quad 68$$

By analogy with equation 67, it follows that:

$$\gamma_2K_1 = K_b \quad 69$$

5.3 Solution for the microscopic constants and population parameters in terms of graphical parameters – partial receptor inactivation

When the transducer slope factor in the operational model is equivalent to one ($m = 1$), the microscopic constants and population parameters of the operational model for allosterism can be expressed in terms of the graphical parameters of the concentration-response curves. In this section, we summarize these equations for the case involving partial receptor inactivation.

First, we derive equations for the graphical parameters of the concentration-response curves in terms of the population parameters of the operational model for allosterism (equation 17). The graphical parameters (see Figure 10) include the basal response in the absence of agonist (B_1), the EC_{50} value (EC_1) and maximal response of the agonist (E_1). In the presence of arbitrary and maximally effective concentrations of allosteric modulator, the corresponding variables are $B_{4\text{-}i}$, $EC_{4\text{-}i}$ and $E_{4\text{-}i}$ and B_4, EC_4 and E_4, respectively. For the conditions of partial receptor inactivation or reduced receptor expression and no allosteric ligand, the analogous parameters are denoted as B_2, EC_2 and E_2, respectively. For the condition of arbitrary and maximally effective concentrations of allosteric modulator after partial receptor inactivation or reduced receptor expression, the analogous parameters are $B_{3\text{-}i}$, $EC_{3\text{-}i}$ and $E_{3\text{-}i}$ and B_3, EC_3 and E_3, respectively.

The ratio of $E_{4\text{-}i}/EC_{4\text{-}i}$ divided by that measured in the absence of allosteric modulator (E_1/EC_1) sheds light on the influence of allosteric modulators on the concentration-response curve of an agonist when $m = 1$ (F. J. Ehlert, 2005). Figure 10*b* shows a plot of this ratio ($E_{4\text{-}i}EC_1/E_1EC_{4\text{-}i}$) against the concentration of allosteric modulator. When $m = 1$, this ratio is equivalent to the product of the scalar changes in the affinity and efficacy of the orthosteric ligand cause by the allosteric ligand (RA). The concentration of modulator causing a half-maximal increase in this ratio is defined as A_{50}, which is more easily appreciated on the plot of the normalized RA value (RA_{norm}) in Figure 10*c*. RA_{norm} is defined below (equation 89). For the condition of partial receptor inactivation or reduced receptor expression, the analogous RA estimate ($E_{3\text{-}i}EC_2/E_2EC_{3\text{-}i}$)

is denoted as *RAQ* (Figure 10*e*), its normalized value as RAQ_{norm} (Figure 10*f*), and the concentration of allosteric ligand causing a half-maximal change in *RAQ* as AQ_{50}.

The equation for the maximal response to an agonist (E_{max}) without receptor inactivation (q = 1) can be derived by taking the limit of equation 17 as D approaches infinity for the condition of a lack of allosteric modulator (E_1, $A = 0$) and the presence of a maximally effective concentration of allosteric modulator (E_4, limit as A approaches infinity):

$$E_1 = \frac{M_{sys}\tau}{1+\tau} \quad 70$$

$$E_4 = \frac{\gamma_1 M_{sys}\tau}{\alpha+\gamma_1\tau} \quad 71$$

For the condition of partial receptor inactivation ($0 < q < 1$), the analogous limits (equation 25) yield the parameters E_2 and E_3:

$$E_2 = \frac{M_{sys}(q(\tau-\tau_{sys})+\tau_{sys})}{1+q(\tau-\tau_{sys})+\tau_{sys}} \quad 72$$

$$E_3 = \frac{M_{sys}(\gamma_1 q\tau-\alpha(q-1)+\tau_A)}{\gamma_1 q\tau+\alpha(1+q(1+\tau_A))} \quad 73$$

For the condition of an arbitrary concentration of allosteric modulator in the absence (E_{4i}) and presence of partial receptor inactivation (E_{3i}), the appropriate limits yield the following equations:

$$E_{4i} = \frac{M_{sys}\tau(1+A\gamma_1 K_2)}{1+\tau+AK_2(\alpha+\gamma_1\tau)} \quad 74$$

$$E_{3i} = \frac{M_{sys}\Big((1+A\alpha K_2)(A\tau_A K_2+\tau_{sys})+q\Big((1+AK_2)(1+A\alpha\gamma_1 K_2)\tau-(1+A\alpha K_2)(A\tau_A K_2+\tau_{sys})\Big)\Big)}{1+q\tau+AK_2\Big(1+q\big(\tau(1+\gamma_1+A\gamma_1 K_2-\tau_A)\big)+\tau_A+\alpha\Big(1+AK_2\big(1+T_A(1-q)\big)\Big)\Big)+\tau_{sys}-(A\alpha K_2(q-1)+q)\tau_{sys}} \quad 75$$

The constitutive response of the receptor, in the absence (B_1) or presence (B_2) of partial receptor inactivation, can be derived by solving equation 17 for the condition of $D = 0$ in the absence of allosteric ligand ($A = 0$):

$$B_1 = B_2 = \frac{M_{sys}\tau_{sys}}{1+\tau_{sys}} \quad 76$$

In the absence of orthosteric agonist, the response to a maximally effective concentration of allosteric ligand, in the absence (B_4) or presence (B_3) of partial receptor inactivation, can be derived by taking the limit as A approaches infinity when $D = 0$:

$$B_3 = B_4 = \frac{M_{sys}\tau_A}{1+\tau_A} \quad 77$$

The corresponding approach yields the equation for an arbitrary concentration of allosteric modulator, in the absence (B_{4i}) and presence (B_{3i}) of partial receptor inactivation:

$$B_{3i} = B_{4i} = \frac{M_{sys}(A\tau_A K_2+\tau_{sys})}{1+AK_2(1+\tau_A)+\tau_{sys}} \quad 78$$

EC_{50} values of the orthosteric agonist can be derived from the following relationship for the half-maximal response ($response_{50}$):

$$response_{50} = 0.5(B + E_{max}) \quad 79$$

in which B denotes the basal response measured in the absence of orthosteric ligand. If the operational model (Equation 25) is substituted for $resonse_{50}$ and the appropriate equations are substituted for the basal response (B_1, B_3 or $B_{3\text{-}i}$) and E_{max} (E_1, E_2, E_3, $E_{3\text{-}i}$, E_4 or $E_{4\text{-}i}$), then solving the equation for D yields an equation for the EC_{50} value of the orthosteric ligand. These are given next for the condition of no allosteric ligand,

$$EC_1 = \frac{1+\tau_{sys}}{K_1(1+\tau)} \quad 80$$

no allosteric ligand and after partial receptor inactivation,

$$EC_2 = \frac{1+\tau_{sys}}{K_1\left(1+q\tau+\tau_{sys}(1-q)\right)} \quad 81$$

in the presence of a maximally effective concentration of allosteric modulator,

$$EC_4 = \frac{1+\tau_A}{K_1(\alpha+\gamma_1\tau)} \quad 82$$

in the presence of an arbitrary concentration of allosteric modulator,

$$EC_{4i} = \frac{1+AK_2(1+\tau_A)+\tau_{sys}}{K_1(1+\tau+AK_2(\alpha+\gamma_1\tau))} \quad 83$$

in the presence of partial receptor inactivation and a maximally effective concentration of allosteric modulator,

$$EC_3 = \frac{1+\tau_A}{K_1\left(q\gamma_1\tau+\alpha\left(1+\tau_A(1-q)\right)\right)} \quad 84$$

and finally, in the presence of receptor inactivation and an arbitrary concentration of allosteric modulator

$$EC_{3i} = \frac{(1+AK_2)\left(1+AK_2(1+\tau_A)+\tau_{sys}\right)}{K_1\left(1+q\tau+AK_2\left(1+q(\tau(1+\gamma_1+A\gamma K_2)-\tau_A)+\tau_A+\alpha\left(1+AK_2\left(1+T_A(1-q)\right)\right)\right)+\tau_{sys}-(A\alpha K_2(q-1)+q)\tau_{sys}\right)} \quad 85$$

The product of the changes in the affinity and efficacy of the orthosteric ligand caused by the allosteric ligand (*RA*) is a useful measure of allosteric effects. When the transducer slope factor in the operational model is equal to one ($m = 1$), the *RA* value is given by the following equation for the case of no receptor inactivation:

$$RA = \frac{E_{4i}EC_1}{E_1EC_{4i}} \quad 86$$

Substituting in equations 70, 74, 80 and 83 for the E_{max} (E_1 and E_{4i}) and EC_{50} (EC_1 and EC_{4i}) values yields an equation for the *RA* value for the case of no receptor inactivation ($q = 1$):

$$RA = \frac{(1+A\gamma_1K_2)(1+\tau_{sys})}{1+AK_2(1+\tau_A)+\tau_{sys}} \quad 87$$

Taking the limit of this equation as *A* approaches infinity yields the *RA* value at a maximally effective concentration of allosteric ligand (RA_{max})

$$RA_{max} = \frac{\gamma_1(1+\tau_{sys})}{1+\tau_A} \quad 88$$

The normalized RA (RA_{norm}) value is defined as:

$$RA_{norm} = \frac{RA-1}{RA_{max}-1} \quad 89$$

Using equations 87 – 89, the normalized *RA* value can be expressed in terms of population parameters:

$$RA_{norm} = \frac{AK_2(1+\tau_A)}{1+\tau_{sys}+AK_2(1+\tau_A)} \quad 90$$

This equation shows that if the modulator lacks activity by itself ($\tau_A = 0$) and there is a lack of constitutive activity ($\tau_{sys} = 0$), then RA_{norm} is a measure of receptor occupancy by the allosteric modulator. An analogous relationship has be shown for the normalized change in binding affinity caused by an allosteric ligand (Lazareno & Birdsall, 1995). Solving equation 89 for $RA_{norm} = 0.5$ yields the concentration of allosteric ligand that causes a half-maximal change in RA_{norm} (A_{50}):

$$A_{50} = \frac{1+\tau_{sys}}{K_2(1+\tau_A)} \quad 91$$

Note that A_{50} is equivalent to $1/K_2$ whenever both τ_{sys} and τ_A are equivalent to zero.

A useful parameter related to the *RA* value, but measured under conditions of receptor inactivation (*RAQ*), can be defined for the case of $m = 1$:

$$RAQ = \frac{E_{3i}EC_2}{E_2EC_{3i}} \quad 92$$

Substituting in the appropriate equations (74, 77, 83 and 87) for E_{max} and EC_{50} yields:

$$RAQ = \frac{(1+\tau_{sys})\left((1+A\alpha K_2)(A\tau_A K_2+\tau_{sys})+q\left((1+AK_2)(1+A\gamma_1 K_2)\tau-(1+A\alpha K_2)(A\tau_A K_2+\tau_{sys})\right)\right)}{(1+AK_2)\left(1+\tau_{sys}+AK_2(1+\tau_A)\right)\left(\tau_{sys}+q(\tau-\tau_{sys})\right)} \quad 93$$

Taking the limit of this equation as *A* approaches infinity yields the *RAQ* value at a maximally effective concentration of allosteric ligand (RAQ_{max}):

$$RAQ_{max} = \frac{(q\gamma_1\tau-\alpha(q-1)\tau_A)(1+\tau_{sys})}{(1+\tau_A)\left(\tau_{sys}+q(\tau-\tau_{sys})\right)} \quad 94$$

The normalized *RAQ* value (RAQ_{norm}) is defined in a manner analogous to that of RA_{norm}:

$$RAQ_{norm} = \frac{RAQ-1}{RAQ_{max}-1} \quad 95$$

After substituting in equations 94 and 95 for RAQ and RAQ_{max}, this equation can be used to solve for K_2 as described below.

Having defined the graphical parameters of the relevant concentration-response curves in terms of the population parameters, we can solve these equations for the population parameters:

$$M_{sys} = \frac{E_1EC_2 - E_2EC_1}{EC_2 - EC_1} \quad 96$$

$$\tau_{sys} = \frac{B_1(EC_2 - EC_1)}{EC_2(E_1 - B_1) + EC_1(B_1 - E_2)} \quad 97$$

$$q = \frac{EC_1(E_2 - B_1)}{EC_2(E_1 - B_1)} \quad 98$$

$$\tau = \frac{E_1(EC_2 - EC_1)}{EC_1(E_1 - E_2)} \quad 99$$

$$K_1 = \frac{E_1 - E_2}{EC_2(E_1 - B_1) + EC_1(B_1 - E_2)} \quad 100$$

$$\tau_A = \frac{B_3(EC_2 - EC_1)}{E_1EC_2 - E_2EC_1 - B_3(EC_2 - EC_1)} \quad 101$$

$$K_2 = \frac{EC_1(E_2 - B_3) + EC_2(B_3 - E_1)}{A_{50}\left(EC_1(E_2 - B_3) + EC_2(B_1 - E_1)\right)} \quad 102$$

$$\gamma_1 = \frac{E_4EC_1\left(E_1EC_2 - E_2EC_1 + B_1(EC_1 - EC_2)\right)}{E_1EC_4\left(E_1EC_2 - E_2EC_1 + B_3(EC_1 - EC_2)\right)} \quad 103$$

$$\alpha = \frac{\left(E_1EC_2 - E_2EC_1 + B_1(EC_1 - EC_2)\right)\left(E_1EC_2 - E_2EC_1 + E_4(EC_1 - EC_2)\right)}{EC_4(E_1 - E_2)\left(E_1EC_2 - E_2EC_1 + B_3(EC_1 - EC_2)\right)} \quad 104$$

Equations for α, γ_1 and K_2 can be derived that do not involve graphical parameters for allosteric modulation in the absence of receptor inactivation (i.e., not involving A_{50}, E_4 and EC_4):

$$\alpha = \frac{\left(E_1EC_2 - E_2EC_1 + B_1(EC_1 - EC_2)\right)\left(E_1EC_2 - E_2EC_1 + E_3(EC_1 - EC_2)\right)}{EC_3(E_1 - E_2)\left(E_1EC_2 - E_2EC_1 + B_3(EC_1 - EC_2)\right)} \quad 105$$

$$\gamma_1 = \frac{N_1(N_2 + N_3 + N_4)}{E_1(B_1 - E_2)\left(E_1EC_2 - E_2EC_1 + B_3(EC_1 - EC_2)\right)^2 EC_3} \quad 106$$

in which

$$N_1 = \left(E_1EC_2 - E_2EC_1 + B_1(EC_1 - EC_2)\right) \quad 107$$

$$N_2 = -B_3(B_1 - E_2)(E_2 - E_3)EC_1^2 \quad 108$$

$$N_3 = (B_1B_3(E_1 + E_2) + (B_3 + E_1)E_2E_3 - 2B_3E_1E_2 - B_1(B_3 + E_2)E_3)EC_1EC_2 \quad 109$$

$$N_4 = E_1(E_1 - B_1)(B_3 - E_3)EC_2^2 \quad 110$$

To derive an equation for K_2 that does not include A_{50}, E_4 and EC_4, the substituted form of equation 95 is set equal to 0.5 and the variable A is replaced with AQ_{50}. The latter represents the concentration of allosteric ligand causing a half-maximal change in RAQ_{norm}. The resulting equation is solved for K_2 to yield:

$$K_2 = \frac{-B+\sqrt{B^2-4C}}{2} \quad 111$$

in which,

$$B = \frac{(\tau_A+\tau_{sys})\left(\alpha(q-1)(2+\tau_A)(1+\tau_{sys})+(1+\tau_A)(2+\tau_{sys})+q\left(\tau\left(\gamma_1(1+\tau_{sys})-1-\tau_A\right)-(1+\tau_A)(2+\tau_{sys})\right)\right)}{AQ_{50}(1+\tau_A)\left(\alpha\tau_A(1+\tau_{sys})-\tau_{sys}(1+\tau_A)+q\left(\tau_{sys}-\tau_A\left(\alpha+\tau_{sys}(\alpha-1)+\tau\left(\gamma_1(1+\tau_{sys})-1-\tau_A\right)\right)\right)\right)} \quad 112$$

$$C = -\frac{1+\tau_{sys}}{AQ_{50}^2(1+\tau_A)} \quad 113$$

Equations 97, 98, 99, 101, 105 and 106 can be substituted for the corresponding population parameters in this equation so that ultimately, K_2 is expressed in terms of graphical parameters.

To solve the microscopic constants in terms of graphical parameters, equations 55 – 61 and 63 and 66 are first solved to express the microscopic constants in terms of population parameters:

$$K_a = \frac{K_1\tau_A(\gamma_1-\alpha)}{\gamma_1(\tau_A-\tau_{sys})} \quad 114$$

$$K_b = \frac{K_1\tau}{\tau_{sys}} \quad 115$$

$$K_e = \frac{K_2(\gamma_1\tau-\alpha\tau_A)}{\gamma_1(\tau-\tau_{sys})} \quad 116$$

$$K_f = \frac{K_2\tau_A}{\tau_{sys}} \quad 117$$

$$K_{q-obs} = \frac{\tau_{sys}(\alpha\tau_A-\gamma_1\tau_{sys})}{\gamma_1(\tau-\tau_{sys})(\tau_{sys}-\tau_A)} \quad 118$$

$$K_{E-obs} = \frac{\alpha\tau_A-\gamma_1\tau_{sys}}{\gamma_1\tau\tau_A+\alpha\tau_A\tau_{sys}-\gamma_1\tau_{sys}(\tau+\tau_A)} \quad 119$$

Equations for K_b and $K_{q\text{-}obs}$ can be derived that do not depend on a measureable value of τ_{sys}:

$$K_b = \frac{\gamma_1 K_1 \tau}{\tau_A} \quad (120)$$

$$K_{q-obs} = \frac{\tau_A\left(\alpha\gamma_1\tau\tau_{sys} - \alpha\tau_A^2 + \gamma_1\left(\tau_A\tau_{sys} + \tau\left(\tau_A - \tau_{sys}(1+\gamma_1)\right)\right)\right)}{\gamma_1(\tau-\tau_A)(\gamma_1\tau-\tau_A)(\tau_A-\tau_{sys})} \quad (121)$$

The equations for the population parameters, expressed in terms of the graphical parameters (equations 96 – 106 and 111), can be substituted into the foregoing equations (i.e., 114 – 121) for microscopic constants to express the latter in terms of graphical parameters. In the case of K_b and K_f, the resulting equations are simple:

$$K_b = \frac{E_1}{EC_1B_1} \quad (122)$$

$$K_b = \frac{E_4}{EC_4B_1} \quad (123)$$

$$K_f = \frac{B_3}{A_{50}B_1} \quad (124)$$

5.4 Solution for the K_a and K_{bqs}' values in terms of the graphical parameters of the concentration-response curves of orthosteric and allosteric ligands measured independently – partial receptor inactivation

As described under "Results" the K_{bqs}' value of the most efficacious agonist and the K_a values of all less efficacious ligands in a series can be estimated from the independent concentration-response curves of a group of orthosteric and allosteric ligands. To prove this for the case of m = 1 and including the situation of no measureable constitutive activity, we begin by solving the graphical parameters shown in Figure 10 in terms of microscopic constants.

Although sometimes immeasurably small, the equation for the basal response in the absence of ligands (B_1) can be derived by taking the limit of equation 5 as D approaches 0:

$$B_1 = \frac{M_{sys}K_{q-obs}}{K_{q-obs} + K_{E-obs}(1+K_{E-obs})} \quad (126)$$

Taking the limit of equation 13 as D approaches infinity yields an equation for the maximal response of the most efficacious agonist in a series (standard agonist) for the case of no receptor inactivation (E_1, $q = 1$) and partial receptor inactivation E_2, $1 > q > 0$):

$$E_1 = \frac{(1+K_{q-obs})M_{sys}K'_{bqs}}{K'_a+K'_{bqs}(1+K_{q-obs})(1+K_{E-obs})} \quad 127$$

$$E_2 = \frac{(1+K_{q-obs})M_{sys}K'_{bqs}(q+K_{q-obs})+K'_aK_{q-obs}(1+q)}{K'_a+K'_{bqs}(1+K_{q-obs})(1+K_{E-obs})} \quad 128$$

In these two equations, K_a' and K_{bqs}' denote the K_a and K_{bqs} values of the standard agonist. The EC_{50} values of the standard agonist for the condition of no receptor inactivation (EC_1) and partial receptor inactivation (EC_2, $0 < q < 1$) can be derived by substituting in the appropriate values for B (B_1, equation 126) and E_{max} (E_1 or E_2, equations 127 and 128, respectively) in equation 79 and solving for EC_{50}:

$$EC_1 = \frac{K_{q-obs}+K_{E-obs}(1+K_{q-obs})}{K_{E-obs}\left(K'_a+K'_{bqs}(1+K_{q-obs})(1+K_{E-obs})\right)} \quad 129$$

$$EC_2 = \frac{(1+K_{q-obs})\left(K_{q-obs}+K_{E-obs}(1+K_{q-obs})\right)}{K'_{bqs}K_{E-obs}(1+K_{q-obs})(K_{E-obs}(1+K_{q-obs})+q+K_{q-obs})+K'_a(K_{E-obs}(1+K_{q-obs})+q-qK_{q-obs})} \quad 130$$

The E_{max} value (E_{12}) of an agonist (test agonist) having an efficacy less than that of the standard agonist can be derived by taking the limit of equation 14 as D approaches infinity:

$$E_{12} = \frac{(1+K_{q-obs})M_{sys}K_{bqs}RA_i}{K_a+K_{bqs}(1+K_{q-obs})(1+K_{E-obs})RA_i} \quad 131$$

in which RA_i is defined by equation 15.

The equation for the EC_{50} value of the test agonist can be derived from equation 79 using substitutions for B (equation 126), E_{max} (equation 131) and $response_{50}$ (equation 15):

$$EC_{12} = \frac{K_{q-obs}+K_{E-obs}(1+K_{q-obs})}{K_{E-obs}\left(K_a+K_{bqs}(1+K_{q-obs})RA_i+(1+K_{E-obs})\right)} \quad 132$$

As described previously, RA_i is a measure of the product of affinity and efficacy of an agonist expressed relative to that of a standard agonist. It is also a relative measure of K_b (K_b/K_b') (see

also equation 14). When the transducer slope factor in the operational model is equivalent to 1.0, RA_i is given by the following equation:

$$RA_i = \frac{E_{12}EC_1}{E_1EC_{12}} \quad 133$$

Using this equation for RA_i and the appropriate equations for M_{sys} (equation 96) and q (equation 98), it is possible to solve equations 126 – 132 for K_a and the K_{bqs}':

$$K_a = \frac{EC_1(E_{12}-EC_2)+EC_2(E_1-E_{12})}{EC_1EC_{12}(B_1-E_2)+EC_{12}EC_2(E_1-B_1)} \quad 134$$

$$K_{bqs}{}' = \frac{E_1(EC_2-EC_1)}{EC_1(E_1EC_2+B_1(EC_1-EC_2)-E_2EC_1)} \quad 135$$

5.5 Solution for the microscopic constants and population parameters in terms of graphical parameters – reduced receptor expression

As described in the prior two sections the microscopic constants and population parameters of the operational model for allosterism can be expressed in terms of the graphical parameters of the concentration-response curves when ever the transducer slope factor is equivalent to one. In this and the following sections, we summarize these equations for the case involving reduced receptor expression.

For the case of reduced receptor expression, the graphical parameters of the concentration-response curves, expressed in terms of population parameters, are the same as those described above for partial receptor inactivation with the exception of the following:

$$B_2 = \frac{M_{sys}q\tau_{sys}}{1+q\tau_{sys}} \quad 136$$

$$B_3 = \frac{M_{sys}q\tau_A}{1+q\tau_A} \quad 137$$

$$B_{3i} = \frac{M_{sys}q(A\tau_AK_2+\tau_{sys})}{1+AK_2(1+q\tau_A)+q\tau_{sys}} \quad 138$$

$$E_2 = \frac{M_{sys}q\tau}{1+q\tau} \quad 139$$

$$E_3 = \frac{M_{sys}q\tau\gamma_1}{\alpha+q\tau\gamma_1} \quad 140$$

$$E_{3i} = \frac{M_{sys} q(\tau + AK_2 \tau \gamma_1)}{1 + q\tau + AK_2(\alpha + q\tau\gamma_1)} \quad 141$$

$$EC_2 = \frac{1 + q\tau_{sys}}{K_1(1 + q\tau)} \quad 142$$

$$EC_3 = \frac{1 + q\tau_A}{K_1(\alpha + q\tau\gamma_1)} \quad 143$$

$$EC_{3i} = \frac{\left(1 + AK_2(1 + q\tau_A) + q\tau_{sys}\right)}{K_1\left(1 + q\tau + AK_2(\alpha + q\tau\gamma_1)\right)} \quad 144$$

$$RAQ = \frac{(1 + A\gamma_1 K_2)\left(1 + q\tau_{sys}\right)}{1 + AK_2(1 + q\tau_A) + q\tau_{sys}} \quad 145$$

$$RAQ_{max} = \frac{\gamma_1\left(1 + q\tau_{sys}\right)}{1 + q\tau_A} \quad 146$$

$$RAQ_{norm} = \frac{AK_2(1 + q\tau_A)}{1 + AK_2(1 + q\tau_A) + q\tau_{sys}} \quad 147$$

The equations for the graphical parameters can be solved to yield the population parameters:

$$M_{sys} = \frac{B_1 EC_1(E_1 - E_2) + E_1(E_1 EC_2 - E_2 EC_1)}{E_1(EC_2 - EC_1)} \quad 148$$

$$\tau_{sys} = \frac{B_1 E_1(EC_2 - EC_1)}{(E_1 - B_1)(E_1 EC_2 - E_2 EC_1)} \quad 149$$

$$q = \frac{E_2(E_1 - B_1)E_2 EC_1}{E_1(E_2 - B_2)E_1 EC_2} \quad 150$$

$$\tau = \frac{{E_1}^2(EC_2 - EC_1)}{EC_1(E_1 - B_1)(E_1 - E_2)} \quad 151$$

$$K_1 = \frac{E_1 - E_2}{E_1 EC_2 - E_2 EC_1} \quad 152$$

$$\tau_A = \frac{B_3 {E_1}^2 EC_2(E_2 - B_2)(EC_2 - EC_1)}{E_2 EC_1(E_1 - B_1)\left(E_2 EC_1(B_3 - E_2) + EC_2(E_2(E_1 + B_2 - B_3) - E_1 B_2)\right)} \quad 153$$

$$K_2 = \frac{EC_2(E_2(E_1 + B_2 - B_3) - B_2 E_1) - E_2 EC_1(E_2 - B_3)}{A_{50}(E_2 - B_2)(E_1 EC_2 - E_2 EC_1)} \quad 154$$

$$\gamma_1 = \frac{RAQ_{max}(E_2 - B_2)(E_1 EC_2 - E_2 EC_1)}{E_2 EC_1(B_3 - E_2) + EC_2(E_2(E_1 + B_2 - B_3) - B_2 E_1)} \quad 155$$

$$\alpha = \frac{RAQ_{max} E_2(E_2 EC_1 - E_1 EC_2)\left(E_2 EC_1(E_2 - E_3) + B_2 E_1 EC_2 - E_2 EC_2(B_2 + E_1 - E_3)\right)}{E_3 EC_2(E_1 - E_2)\left(E_2 EC_1(B_3 - E_2) + EC_2(E_2(E_1 + B_2 - B_3) - B_2 E_1)\right)} \quad 156$$

To solve the microscopic constants in terms of graphical parameters, the former are first solved in terms of the population parameters (see equations 114 – 121). Next, the equations for the population parameters, expressed in terms of graphical parameters (97 – 105), are substituted

into equations 114 – 121 so that the microscopic constants can be expressed in terms of graphical parameters. In some instances, the solutions are simple:

$$K_e = \frac{E_2(E_3-B_3)}{AQ_{50}E_3(E_2-B_2)} \quad 157$$

$$K_f = \frac{RAQ_{max}}{AQ_{50}} \quad 158$$

5.6 Solution for the K_a and K_{bqs}' values in terms of the graphical parameters of the concentration-response curves of orthosteric and allosteric ligands measured independently – reduced receptor expression

As described under "Results" the K_{bqs}' value of the most efficacious agonist and the K_a values of all less efficacious ligands in a series can be estimated from the independent concentration-response curves of a group of orthosteric and allosteric ligands. To prove this for the case of reduced receptor expression and including the condition of no measureable constitutive activity, we first solve the graphical parameters shown in Figure 10 in terms of microscopic constants. These solutions are the same as those described above for the case of partial receptor inactivation (Equations 126, 127, 129, 131 – 133) except for the following:

$$E_2 = \frac{qM_{sys}K'_{bqs}(1+K_{q-obs})}{K'_a+K'_{bqs}(1+K_{q-obs})(q+K_{E-obs})} \quad 159$$

$$EC_2 = \frac{qK_{q-obs}+K_{E-obs}(1+K_{q-obs})}{K_{E-obs}\left(K'_a+K'_{bqs}(1+K_{q-obs})(q+K_{E-obs})\right)} \quad 160$$

Using the equation for RA_i (equation 133) and the appropriate equations for M_{sys} (equation 148) and q (equation 150), it is possible to solve equations 126, 127, 129, 131 – 133, 159 and 160 for K_a and the K_{bqs}'. The equation for K_{bqs}' is equivalent to that for the case of partial receptor inactivation (equation 135) and that for K_a is:

$$K_a = \frac{EC_1(E_{12}-EC_2)+EC_2(E_1-E_{12})}{EC_1EC_{12}(B_1-E_2)+EC_{12}EC_2(E_1-B_1)} \quad 161$$

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Figure Captions

Figure 1: *Two-state scheme for receptor-G protein interactions*. The active and inactive states of the receptor and G protein, bound with GDP, are shown. The microscopic affinity constants of the agonist for active and inactive states of the receptor are denoted by K_b and K_a, respectively. The equilibrium between the active and inactive states of the receptor is defined by the constant, $K_{q\text{-}obs}$. The sensitivity constant, $K_{E\text{-}obs}$, defines the relationship between receptor activation and the downstream response.

Figure 2: *Allosteric ternary complex scheme defined at the level of receptor states (a) and the receptor population (b). a*, Two-state scheme for allosteric interactions. The cube of equilibria illustrates the binding of orthosteric (*D*) and allosteric (*A*) ligands to distinct sites on a receptor in equilibrium between active (R_s*) and inactive (R_s) states. The isomerization constant of the unoccupied receptor is denoted by $K_{q\text{-}obs}$. The microscopic affinity constants of the orthosteric (K_b and K_a) and allosteric (K_f and K_e) ligands for the active and inactive states of the receptor,

respectively, are shown. *b*, Population scheme for allosteric interactions. The square of equilibria shows the different types of receptor complexes in the receptor population and the binding of orthosteric and allosteric ligands to them. The observed affinity constant of the orthosteric ligand for the receptor population in the absence of allosteric ligand is denoted by K_1. The analogous constant for the allosteric ligand is denoted by K_2. The constant, α, denotes the mutual scalar effect of each ligand on the observed affinity constant of the other ligand. The constants, ε_{sys}, ε, ε* and ε_A, denote the fractions of the populations of receptor species (*R*, *DR*, *DRA* and *RA*, respectively) in the active state.

Figure 3: *Quaternary complex scheme for allosterism*: The scheme describes the equilibrium between receptor and G protein and their attendant ligands. These include orthosteric (*D*) and allosteric (*A*) ligands for the receptor and guanine nucleotide (*X*) for the G protein. *a*, The two central concentric squares of equilibria represent the quaternary complex scheme of Ehlert (F. J. Ehlert, 2000; F. J. Ehlert & Rathbun, 1990). It has been expanded to include the binding of allosteric ligand. Not all of the possible one-step transitions between receptor complexes are shown, but those that are shown can account for the equilibrium levels of all of the receptor complexes. Some of the transitions not shown are clearly feasible (e.g., *RAGX* ↔ *DRAGX*). *b*, Quaternary complex scheme for allosterism at the level of receptor states. Each plane of equilibria is analogous to that shown in panel *a* except that the receptor and G protein are defined at the level of active (R_s^*, G_s^*) and inactive (R_s, G_s) states as designated to the left of the scheme. Again, only a minimum number of transitions are shown that are sufficient to calculate of the equilibrium levels of the various states of the receptor complexes.

Figure 4: *Simulations of the total stimulus function for a highly efficacious agonist in the presence of a positive allosteric modulator*. The simulations were done using equation 37 in the Appendix with the following values for the log microscopic constants of the agonist (log K_a and log K_b) and allosteric modulator (log K_e and log K_f): 4.0, 8.0, 5.0 and 6.5, respectively. The values of the log isomerization constant (log K_q) of the unoccupied receptor, the ratio of G protein to receptor (G_T/R_T) and log concentration of guanine nucleotide (log *X*) were -4.0, 3.0

and -3.0, respectively. The other microscopic constants, which are described in the Appendix, and their log values for this simulation were log K_g, -5; log K_h, -6; log K_j, -6; log K_k, 2.5; log K_l, 9.0; log K_m, 5.0 and log K_r, -4. The points represent the simulated total stimulus values and the curves represent the global least-squares fit of equation 34 to the data.

Figure 5: *Simulation of the effects of a positive allosteric modulator and reduced receptor expression (a) or partial receptor inactivation (b) on the responses of a highly efficacious agonist*. The values of the parameters of the transducer function of the operational were: M_{sys}, 1.0; K_E, 10^{-2} and m, 1.5. The values of the microscopic constants and other parameters for this simulation are the same as in Figure 4. The plots show simulations of the concentration-response curve of an agonist in the absence and presence of various concentrations of allosteric agonist for the case of a decrease in the population of orthosteric sites to only 5% ($q = 0.05$) by reduced receptor expression (*a*) or partial receptor inactivation (*b*). In addition, a concentration response curve was simulated for control conditions. The points represent the mean values ± SEM of four simulations, and the curves represent the global least squares fit (equation 8).

Figure 6: *Simulation of the effects of an allosteric agonist and reduced receptor expression (a and b) or partial receptor inactivation (c) on the responses of a highly efficacious agonist*. *a*, The concentration-response curves of the orthosteric and allosteric agonists are shown for control conditions. *b*, Simulations of the concentration-response curve of an agonist in the absence and presence of various concentrations of allosteric agonist for the case of the reduced receptor expression to 5% ($q = 0.05$). *c*, The concentration-response curve of the agonist was simulated for control conditions and for the condition of partial receptor inactivation ($q = 0.05$) in the absence and presence of various concentrations of allosteric agonist. The values of the microscopic constants of the orthosteric ligand, the quaternary complex scheme, and the operational model are the same as those in Figure 4. The log values of the microscopic constants (log K_e and log K_f) of the allosteric agonist were 5.0 and 7.2, respectively. The points represent the mean values ± SEM of four simulations, and the curves represent the global least squares fit of equations 11 (*a* and *b*) and 7 (*c*) to the simulated data points.

Figure 7: *Simulation of the effects of reduced receptor expression (a and b) and partial receptor inactivation (c) (q = 0.01) on negative allosteric interactions at a receptor exhibiting constitutive activity*. *a*, The concentration-response curves of the orthosteric and allosteric ligands are shown for control conditions. *b*, The concentration-response curves of the agonist were simulated in the absence and presence of various concentrations of the negative allosteric ligand for the case of reduced receptor expression to only 5% ($q = 0.05$). *c*, The concentration-response curve of the agonist was simulated for control conditions and for the condition of partial receptor inactivation ($q = 0.05$) in the absence and presence of various concentrations of allosteric ligand. The log values of the microscopic constants (log K_a and log K_b) of the orthosteric agonist were 4.0 and 7.0, respectively. The corresponding values (log K_e and log K_f) of the negative allosteric ligand were 6.0 and 5.0, respectively. The values of the constants of the operational model are the same as those in Figure 4, and those of the quaternary complex scheme are also the same as those of Figure 4 except for log K_g, -6.0; log K_k, 1.5 and log K_q, -2.5. The points represent the mean values ± SEM of four simulations, and the curves represent the global least squares fit of equations 11 (*a* and *b*) and 7 (*c*) to the simulated data points.

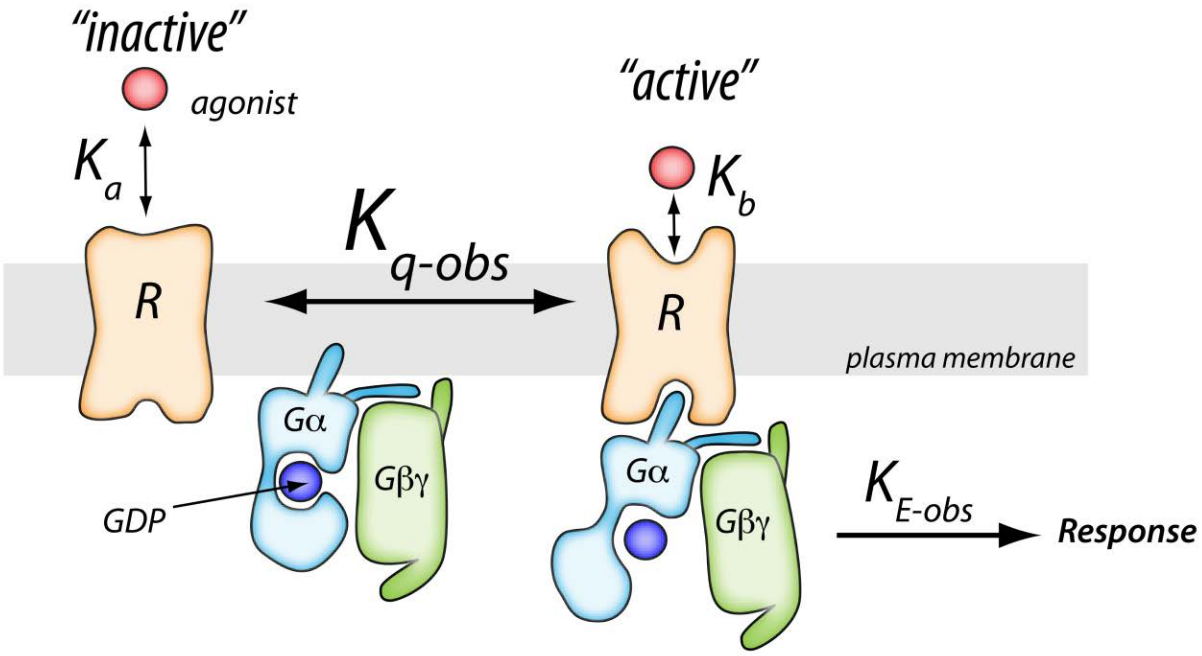
Figure 8: *Simulation of the independent effects of orthosteric and allosteric ligands*. The concentration-response curves of ligands from some of the prior simulations are plotted for the condition of no interacting ligand. The source of the simulated concentration-response curves is indicated in parentheses. *a*, A highly efficacious agonist and an allosteric agonist (Figure 6*a*). *b*, A highly efficacious agonist and an allosteric agonist (Figure 6*c*). c, A highly efficacious agonist and a negative allosteric ligand (Figure 7*a*). *d*, A highly efficacious agonist and a negative allosteric ligand (Figure 7*c*).

Figure 9: *Allosteric modulation of M_1 muscarinic receptor-mediated signaling through a chimeric Gpa1/G$\alpha_{i1,2}$ chimera in yeast (a) and of M_2 muscarinic receptor-mediated inhibition of forskolin-stimulated cAMP accumulation in CHO cells (b)*. *a*, The concentration-response curves of carbachol in the absence and presence of various concentration of BQCA. The data have been estimated from Figure 5A in Canals et al. (2012) and reproduced with permission. *b*,

The concentration-response curves of oxotremorine-M were measured following partial receptor inactivation with 4-DAMP mustard and in the absence and presence of two concentrations of gallamine. A control concentration-response curve was also measured without gallamine or receptor inactivation. The data have been reproduced from Figure 5*f* in Ehlert and Griffin (2008) with permission.

Figure 10: *Graphical parameters for allosteric modulation*: *a*, The EC_{50} value and maximal response of an orthosteric ligand measured in the absence (EC_1 and E_1) and presence of arbitrary ($EC_{4\text{-}i}$ and $E_{4\text{-}i}$) and maximally effective (EC_4 and E_4) concentrations of allosteric modulator are shown. The corresponding values for the basal response in the absence of orthosteric ligand are denoted by B_1, $B_{4\text{-}i}$ and B_4. *b*, The log ratio of $E_{4\text{-}i}EC_1/E_1EC_{4\text{-}i}$ is plotted against the log allosteric ligand concentration. When $m = 1$, the value of this ratio is equivalent to the relative change in the product of affinity and efficacy of the orthosteric agonist caused by the allosteric ligand (*RA*), the maximum value of which is denoted as RA_{max}. *c*, The normalized *RA* value (RA_{norm}) is plotted against the log concentration of allosteric ligand. A_{50} denotes the concentration of allosteric ligand causing a half-maximal change in RA_{norm}. *d*, The EC_{50} value and maximal response of an orthosteric ligand measured after partial receptor inactivation and in the absence (EC_2 and E_2) and presence of arbitrary ($EC_{3\text{-}i}$ and $E_{3\text{-}i}$) and maximally effective (EC_3 and E_3) concentrations of allosteric modulator are shown. The corresponding values for the basal response in the absence of orthosteric ligand are denoted by B_1, $B_{3\text{-}i}$ and B_3. For comparison, the control concentration-response curve of the orthosteric ligand and its associated parameters (EC_1 and E_1) are also shown. *e*, The log ratio of $E_{3\text{-}i}EC_2/E_2EC_{3\text{-}i}$ is plotted against the log allosteric ligand concentration. When $m = 1$, this ratio is denoted as *RAQ*, the maximum value of which is denoted as RAQ_{max}. *f*, The normalized *RAQ* value (RAQ_{norm}) is plotted against the log concentration of allosteric ligand. AQ_{50} denotes the concentration of allosteric ligand causing a half-maximal change in RAQ_{norm}.

Figure 1


"inactive"
agonist
K_a
R
GDP
Gα
Gβγ
$K_{q\text{-}obs}$
"active"
K_b
R
plasma membrane
Gα
Gβγ
$K_{E\text{-}obs}$
Response

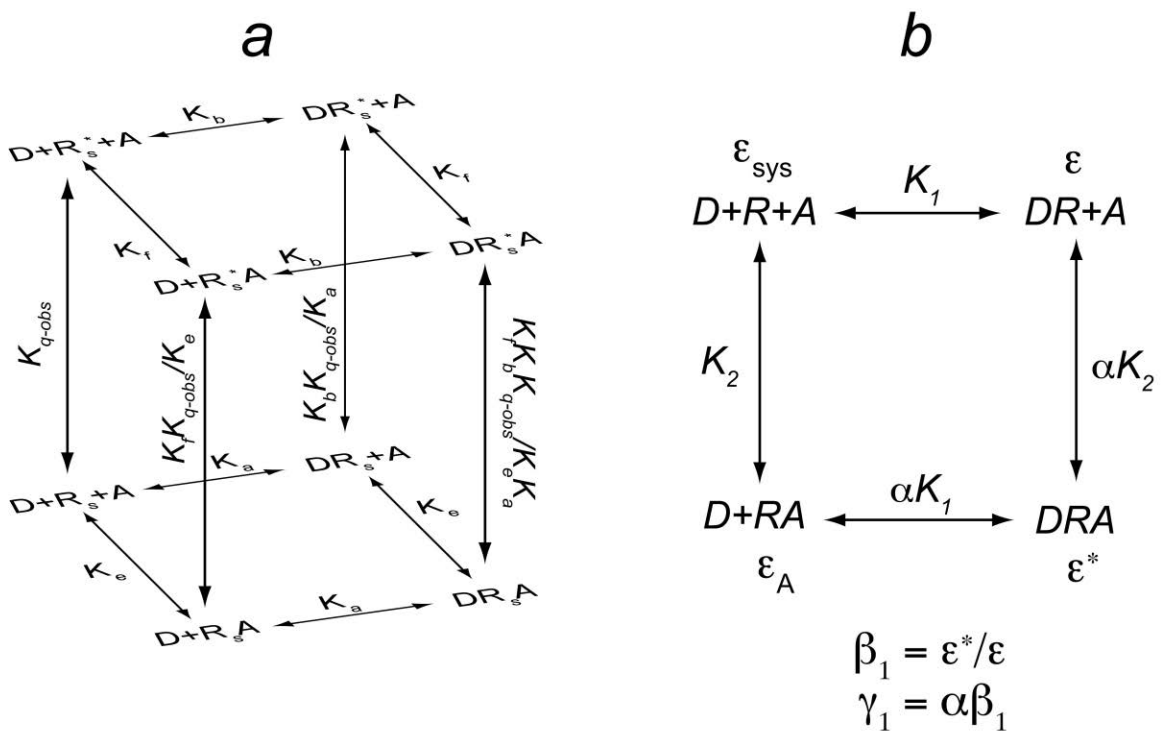

a
b
$K_{q\text{-}obs}$
$K_f K_{q\text{-}obs}/K_e$
$K_b K_{q\text{-}obs}/K_a$
$K_f K_b K_{q\text{-}obs}/K_e K_a$
ε_{sys}
ε
K_1
K_2
αK_2
αK_1
ε_A
ε^*
$\beta_1 = \varepsilon^*/\varepsilon$
$\gamma_1 = \alpha\beta_1$

Figure 2

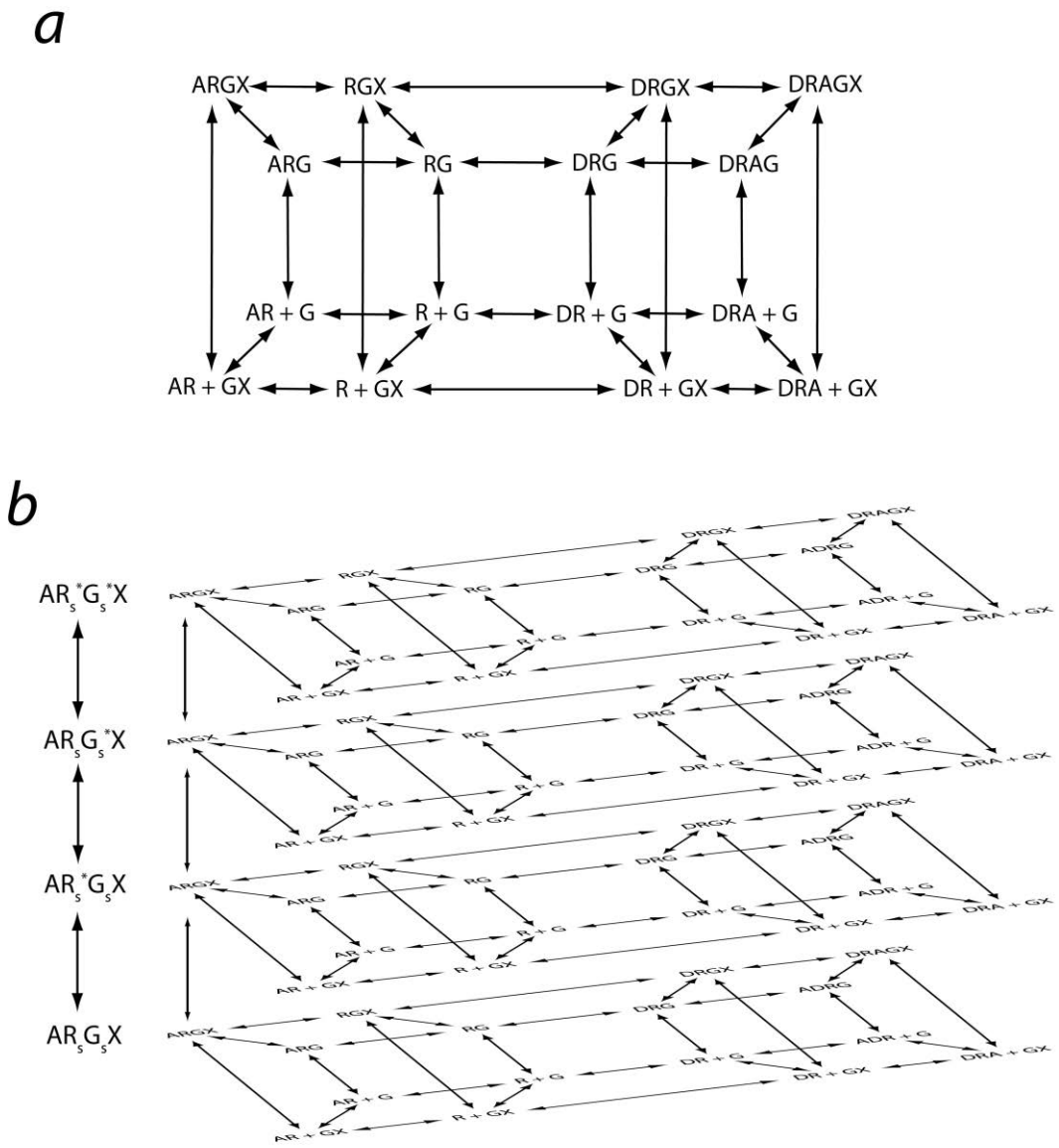

a
ARGX
RGX
DRGX
DRAGX
ARG
RG
DRG
DRAG
AR + G
R + G
DR + G
DRA + G
AR + GX
R + GX
DR + GX
DRA + GX
b
AR*sG*sX
ARsG*sX
AR*sGsX
ARsGsX

Figure 3

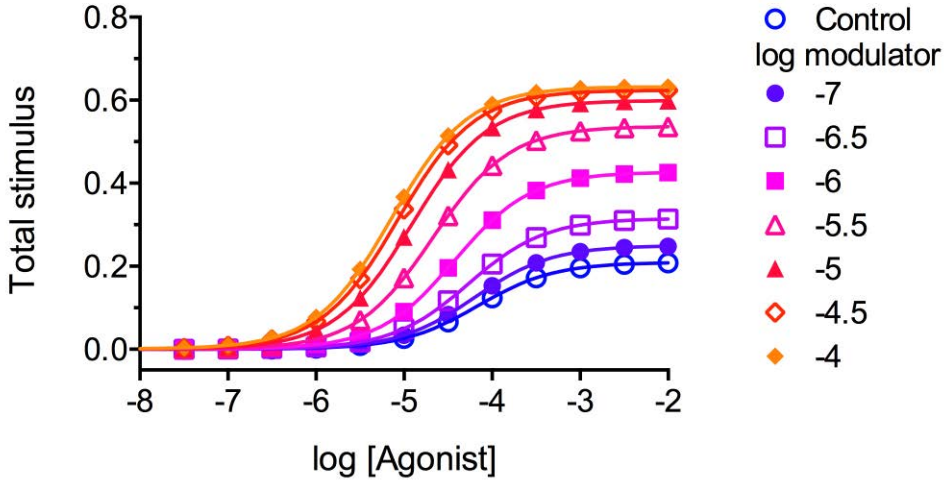

Total stimulus
0.8
0.6
0.4
0.2
0.0
-8
-7
-6
-5
-4
-3
-2
log [Agonist]
Control
log modulator
-7
-6.5
-6
-5.5
-5
-4.5
-4

Figure 4

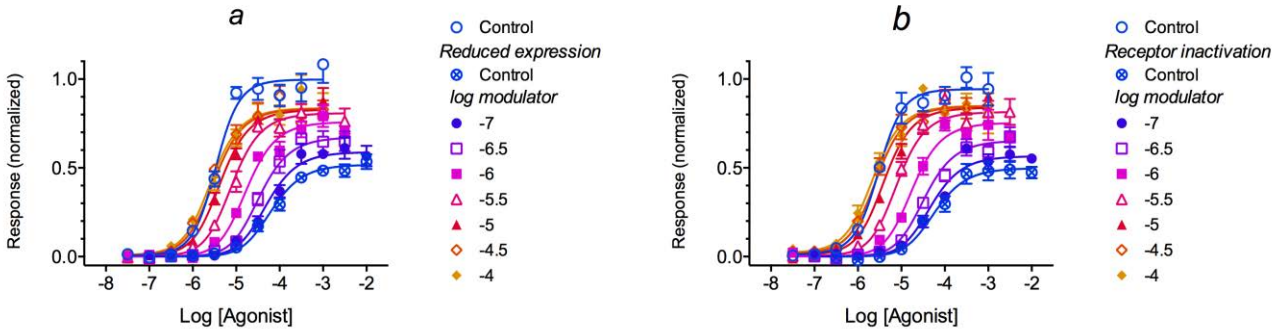

a
Response (normalized)
1.0
0.5
0.0
-8
-7
-6
-5
-4
-3
-2
Log [Agonist]
Control
Reduced expression
Control
log modulator
-7
-6.5
-6
-5.5
-5
-4.5
-4
b
Response (normalized)
1.0
0.5
0.0
-8
-7
-6
-5
-4
-3
-2
Log [Agonist]
Control
Receptor inactivation
Control
log modulator
-7
-6.5
-6
-5.5
-5
-4.5
-4

Figure 5

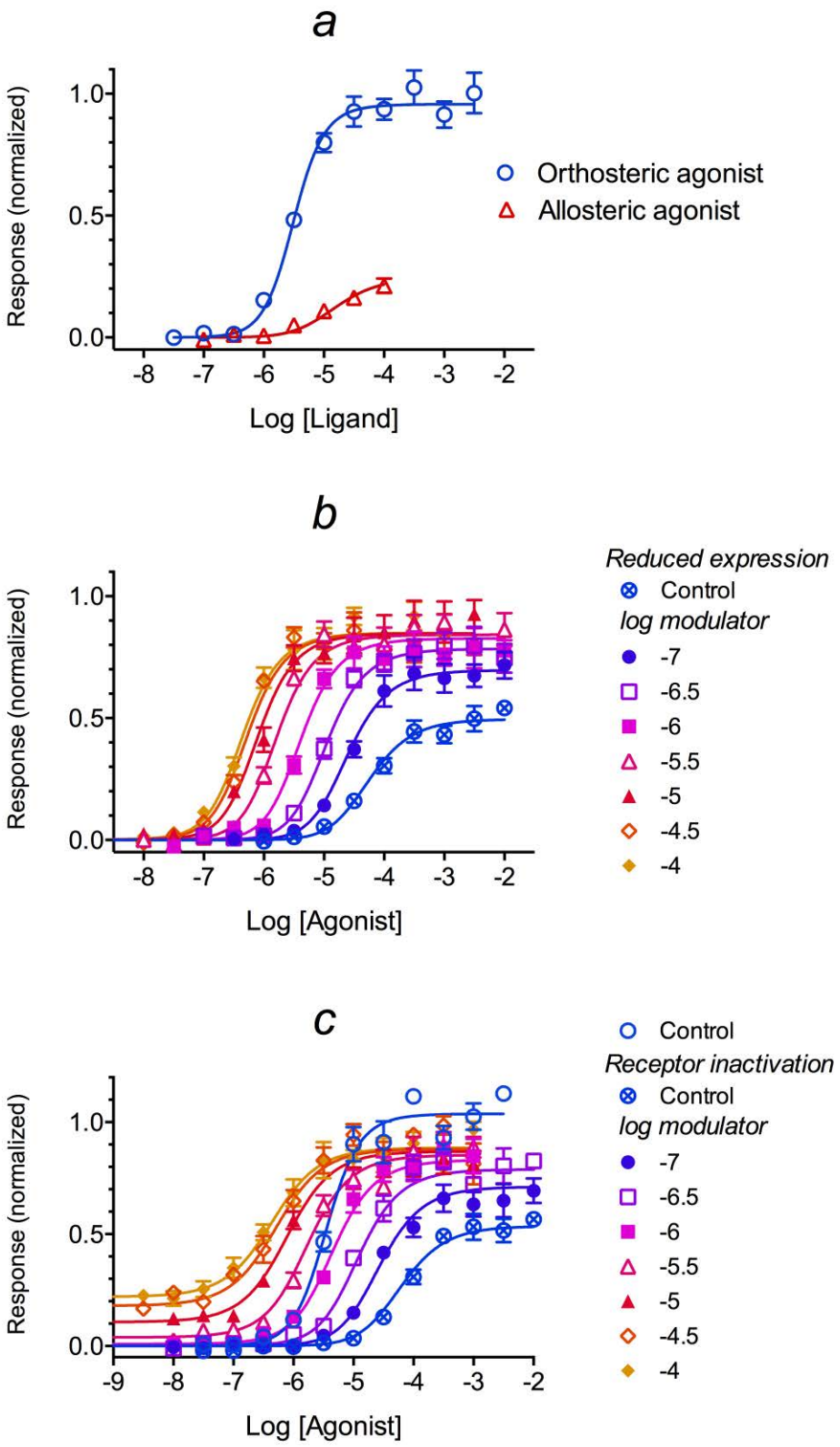

a
Response (normalized)
1.0
0.5
0.0
-8
-7
-6
-5
-4
-3
-2
Log [Ligand]
Orthosteric agonist
Allosteric agonist
b
Response (normalized)
Log [Agonist]
Reduced expression
Control
log modulator
-7
-6.5
-6
-5.5
-5
-4.5
-4
c
Response (normalized)
-9
Log [Agonist]
Control
Receptor inactivation
Control
log modulator
-7
-6.5
-6
-5.5
-5
-4.5
-4

Figure 6

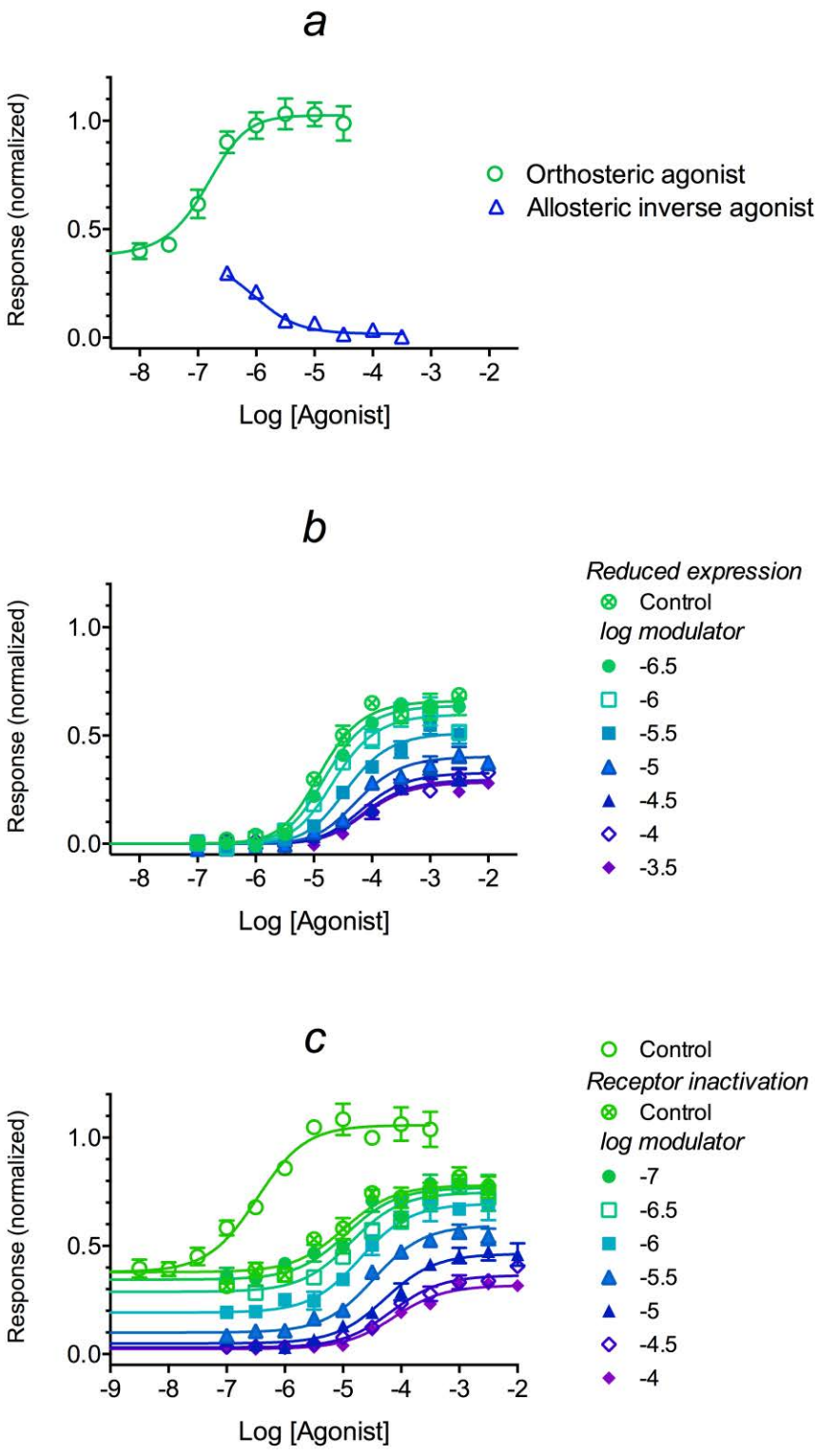

a
Response (normalized)
1.0
0.5
0.0
-8
-7
-6
-5
-4
-3
-2
Log [Agonist]
Orthosteric agonist
Allosteric inverse agonist
b
Response (normalized)
Log [Agonist]
Reduced expression
Control
log modulator
-6.5
-6
-5.5
-5
-4.5
-4
-3.5
c
Response (normalized)
-9
Log [Agonist]
Control
Receptor inactivation
Control
log modulator
-7
-6.5
-6
-5.5
-5
-4.5
-4

Figure 7

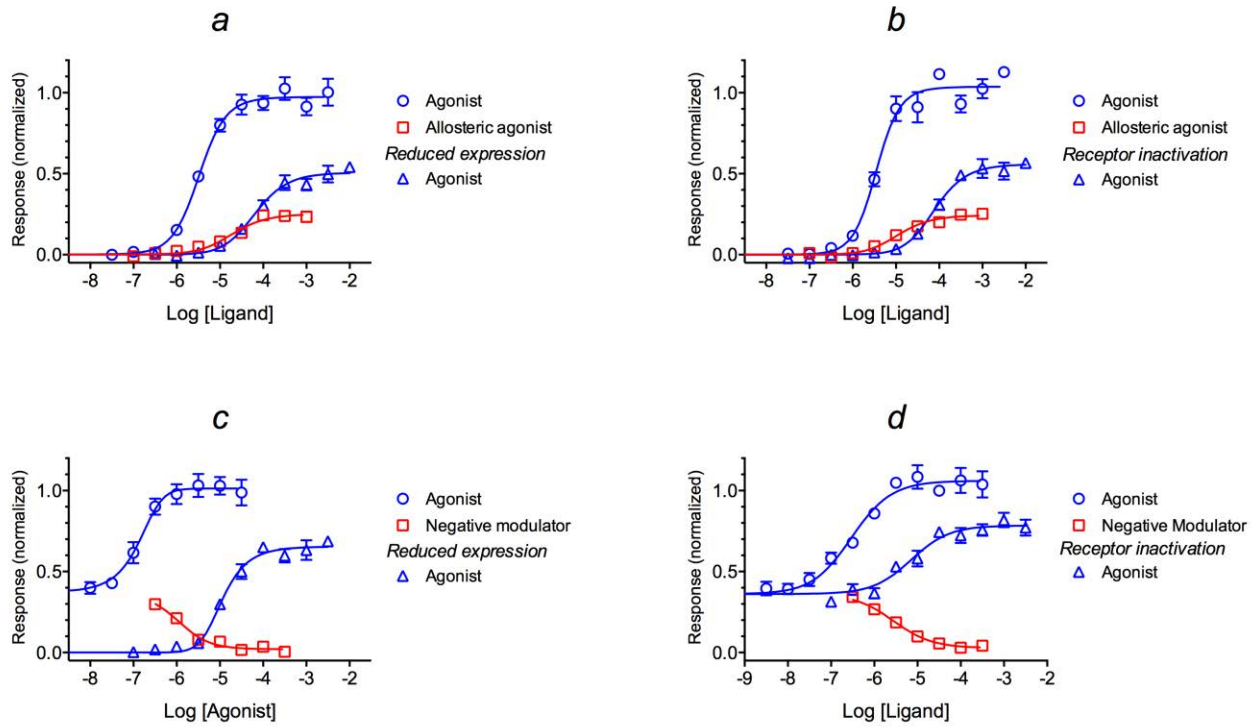

a
Response (normalized)
1.0
0.5
0.0
-8 -7 -6 -5 -4 -3 -2
Log [Ligand]
Agonist
Allosteric agonist
Reduced expression
Agonist
b
Response (normalized)
1.0
0.5
0.0
-8 -7 -6 -5 -4 -3 -2
Log [Ligand]
Agonist
Allosteric agonist
Receptor inactivation
Agonist
c
Response (normalized)
1.0
0.5
0.0
-8 -7 -6 -5 -4 -3 -2
Log [Agonist]
Agonist
Negative modulator
Reduced expression
Agonist
d
Response (normalized)
1.0
0.5
0.0
-9 -8 -7 -6 -5 -4 -3 -2
Log [Ligand]
Agonist
Negative Modulator
Receptor inactivation
Agonist

Figure 8

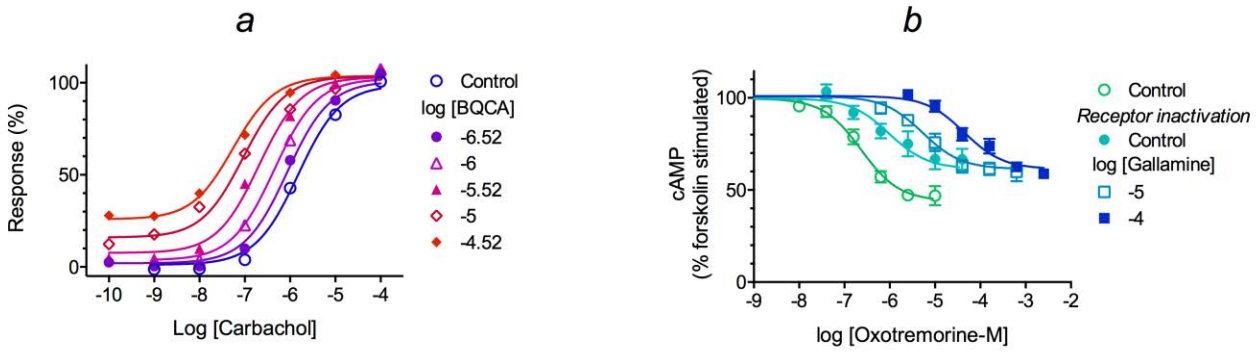

a
Response (%)
100
50
0
-10
-9
-8
-7
-6
-5
-4
Log [Carbachol]
Control
log [BQCA]
-6.52
-6
-5.52
-5
-4.52
b
cAMP
(% forskolin stimulated)
100
50
0
-9
-8
-7
-6
-5
-4
-3
-2
log [Oxotremorine-M]
Control
Receptor inactivation
Control
log [Gallamine]
-5
-4

Figure 9

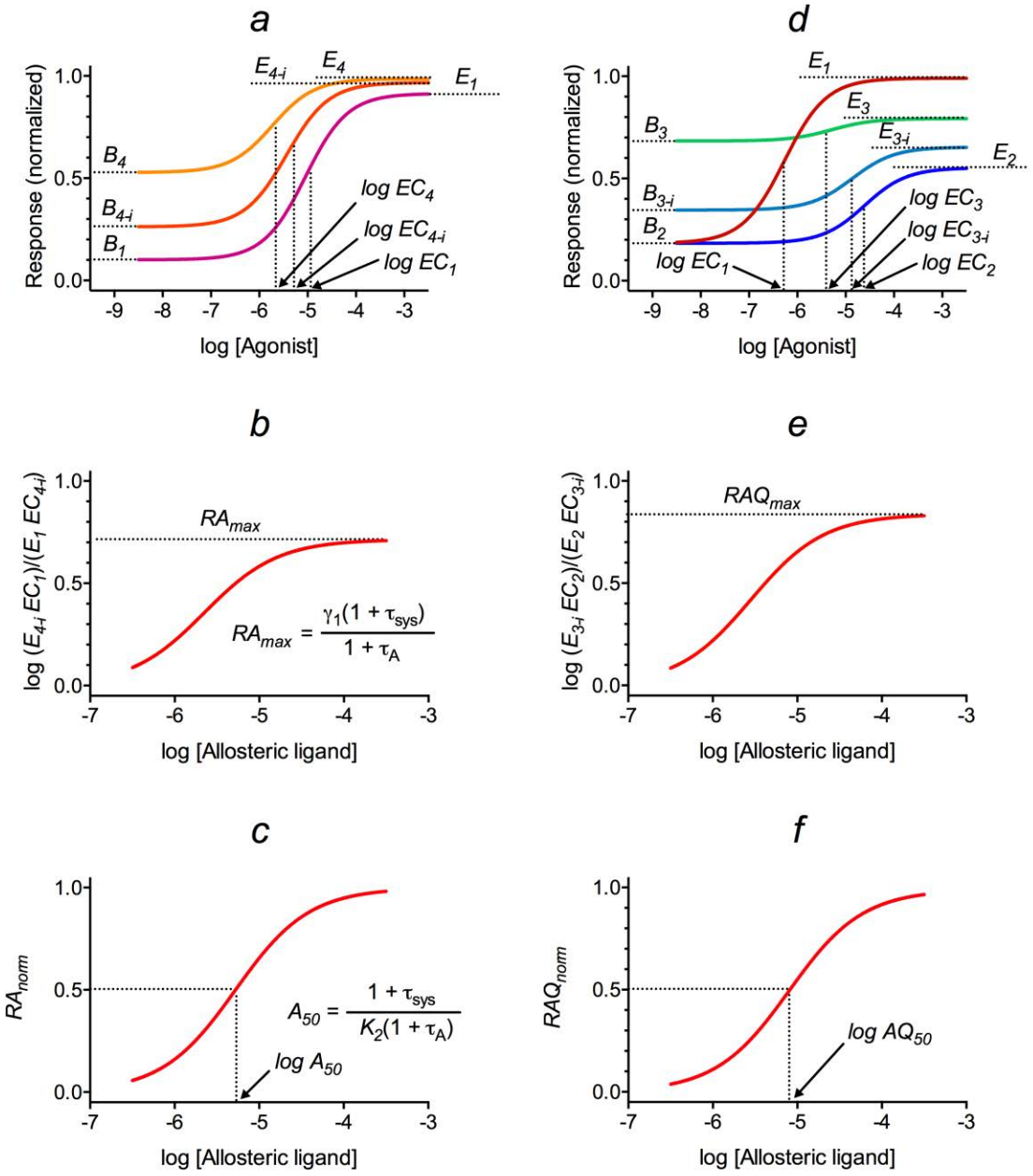

a
Response (normalized)
log [Agonist]
E_{4-i}
E_4
E_1
B_4
B_{4-i}
B_1
log EC_4
log EC_{4-i}
log EC_1
d
E_1
E_3
E_{3-i}
E_2
B_3
B_{3-i}
B_2
log EC_1
log EC_3
log EC_{3-i}
log EC_2
b
log $(E_{4-i}\ EC_1)/(E_1\ EC_{4-i})$
log [Allosteric ligand]
RA_{max}
$RA_{max} = \frac{\gamma_1(1+\tau_{sys})}{1+\tau_A}$
e
log $(E_{3-i}\ EC_2)/(E_2\ EC_{3-i})$
RAQ_{max}
c
RA_{norm}
$A_{50} = \frac{1+\tau_{sys}}{K_2(1+\tau_A)}$
log A_{50}
f
RAQ_{norm}
log AQ_{50}

Figure 10