ORIGINAL RESEARCH

Ligand competition assays indicate allosterism and insufficiency of the ternary complex model

Eyad Qunaibi[1]
Roger Barber[2]

[1]Formerly (during the time of the study): School of Pharmacy, University of Houston, Houston, TX. At present: School of Pharmacy, Applied Sciences University, Amman, Jordan; [2]Formerly (during the time of the study): Department of Integrative Biology and Pharmacology, University of Texas/Houston, Houston, TX, USA

Correspondence: Eyad Qunaibi
Department of Clinical Pharmacy and Therapeutics, Faculty of Pharmacy, Applied Sciences University, PO Box 23002, Amman 11115, Jordan
Tel 00962-79529-6706
Fax 00962-64775142
Email eyadqunaibi@yahoo.com

Background: Many researchers have tried to correlate characteristics of ligand binding at G-protein–coupled receptor (GPCR) with ligand efficacy. The ternary complex model (TCM) is the traditional model for explaining the equilibrium of agonist-GPCR-G-protein interaction, and the effect of this interaction on agonist efficacy. However, no consistent correlation has been proven for various binding-efficacy data, so several extensions of the model have been proposed. These extensions are of descriptive value but their validity cannot be verified by binding-efficacy correlations. Therefore, we developed a novel approach to validate the TCM and its extensions.

Methods: We simulated the predictions of the TCM for relationships within binding parameters. According to the TCM, an increase in the difference between high and low agonist affinities for a receptor (ie, greater K_L/K_H) should be accompanied by stability or an increase in the fraction of receptors bound to the agonist with high affinity ($\boldsymbol{R}_H$). To validate these predictions we performed ligand competition experiments for a set of β_2-adrenergic receptor (AR) agonists and analyzed the resulting binding data as well as data taken from relevant literature.

Results: No smooth relationship exists between $\boldsymbol{R}_H$ and K_L/K_H in our or others' data, indicating the insufficiency of the TCM and its extensions. We introduce the allosteric modulators model as an alternative.

Conclusions: To our knowledge, this is the first paper in which insufficiency of the TCM and its extensions based on binding data are shown, and the first in which the presence of allosteric modulators of ligand affinity is proven to be a necessity for explaining binding data at GPCRs.

Keywords: G-protein-coupled receptor, ternary complex model, allosteric modulators model, fraction of high affinity receptors, ratio of high and low receptor affinities for agonist, binding-functional correlations

Traditionally, ligand-binding assays have been used as a first line filter for the process of selecting new chemical entities (NCEs) that have high affinity for a receptor of interest, and for predicting their efficacy at that receptor. A wide variety of responses beyond the agonist-receptor level have been used to express agonist efficacy at guanyl nucleotide-binding protein (G-protein) coupled receptors. This involves, for example, agonist-induced binding of GTP to G-protein, adenylate cyclase activation, and inositol phosphate production.

Introduction of the ternary complex model (TCM) gave the theoretical basis for trying to correlate ligand efficacy with relative affinities of the ligand for receptors uncoupled versus coupled to G-protein (K_L/K_H) (where K_L is the dissociation constant for binding of agonist to low affinity receptor sites and K_H is the dissociation constant

for binding of agonist to high affinity receptor sites) or, less commonly, the fraction of receptors with high affinity for the ligand (R_H or the fraction of receptors binding the agonist with high affinity at saturating agonist concentrations) in numerous studies.[1–28] However, these correlations have varied from highly significant to completely absent. Several extensions and assumptions have been proposed that accommodate, to some extent, this variability in correlation.[2,17,21,29–33]

One has to bear in mind, however, that the very core concept in the TCM of a ligand-receptor-G protein hierarchy does not put limits on the binding-efficacy correlation; ie, it does not predict a sole or even a restricted range of correlations, disproving that which invalidates the model itself. This precludes using binding-functional correlations to validate the TCM. For example, one can hypothesize that the receptor conformation induced by an agonist to couple to G-protein (the active conformation) is the same for all agonists. By this hypothesis, agonists differ in the fraction of receptors which they put in the active conformation. One could also hypothesize that higher stability of the ternary complex is equal to forming more of the active conformation which, therefore, causes more activation of G-protein and of the downstream effector system. It follows that the agonist which has a higher log K_L/K_H should have a higher efficacy in activating an effector system. Notice that these assumptions are not consequences of the TCM but limitations put on the model to enable deriving relationships between binding parameters and efficacy.

If, however, an agonist with a higher log K_L/K_H is found experimentally to be less efficacious, then explanations can be made that challenge the assumptions added to the TCM but not the TCM per se. For example, it can be said that different conformations are induced by the different agonists.[2,29,30,33] In this context, an agonist with a higher log K_L/K_H can induce a conformation that couples highly to G-protein but activates it less efficiently than the conformation induced by an agonist with a lower log K_L/K_H. Alternatively, it can be said that all agonists induce the same type of conformation that couples to G-protein, but that the receptor then needs to switch to another conformation that activates G-protein. In this context, it can be postulated that an agonist with a higher log K_L/K_H will be less efficacious in activating adenylate cyclase (A/C), not because it cannot induce coupling of receptor to G-protein, but because the coupling it induces is so tight that the receptor cannot switch to the form which activates G-protein.[2]

The above-clarified fluidity of the binding-functional correlations precludes using them to validate the sufficiency

of the TCM and its extensions and modifications. The TCM extensions are valuable in incorporating inevitable observations (like constitutive activity) and accommodating broadly variable data. To validate a model, however, it is not enough to show that it 'accommodates' data. Rather, its 'predictions' need to be proven. The interplay of the many equilibrium constants in the TCM extensions combined with the modifications (like different conformations and promiscuity) gives countless possibilities of efficacy outcomes rather than a single testable prediction.

In this paper, we introduce a novel approach to test the sufficiency of the TCM and its extensions. We show that, unlike with binding-functional correlations, the TCM predicts definite relationships within the binding parameters. We simulated these relationships and ran accurate binding experiments for a series of β_2-adrenergic receptor (β_2-AR) agonists, analyzing binding data for various GPCRs in the literature. We then compared our and others' data with the simulations and formed conclusions on the sufficiency of the TCM.

Materials and methods
Simulation of the relationship within binding parameters

We here simulate the relationship between R_H and K_L/K_H according to the TCM. Following are the equilibrium and equations that describe the ternary complex model:
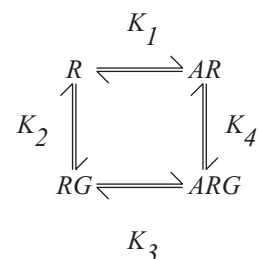
$$K_1 = \frac{[A][R]}{[AR]}, K_2 = \frac{[R][G]}{[RG]}, K_3 = \frac{[RG][A]}{[ARG]}, K_4 = \frac{[AR][G]}{[ARG]}$$

Where R is the receptor, A is the agonist, and G is the G-protein heterotrimer. We will refer to the association of the receptor with the agonist as binding and the association of the receptor with G-protein as coupling in the present study.

According to the TCM, the receptors that are coupled to G-protein have a high affinity for agonist (K_H) while those that are not coupled have a low affinity (K_L). Experimentally, incremental concentrations of this agonist can be incubated with a receptor system that is prebound to an antagonist and the curve of antagonist displacement by the agonist analyzed.

From this analysis we can determine K_L, K_H, and R_H, the apparent fraction of receptors that should bind the agonist with high affinity at saturating agonist concentrations.

To define the predictions of the TCM for the relationship between K_L/K_H and R_H, we elaborate first on the affinity constants in the equilibrium above (K_1-K_4) and explain how they relate to the affinity constants (K_L and K_H) obtained from a 2-site analysis of ligand competition curves. In the TCM equilibrium, a low K_2 value indicates that coupling of receptors to G-protein in the absence of agonist is highly likely, and is therefore agonist-independent (constitutive). A high K_2 value, in contrast, indicates that coupling of receptors to G-protein in the absence of agonist is highly unlikely, and is therefore agonist-dependent. K_L of 2-site analysis is equal to K_1 according to TCM. On the other hand, correlating K_H of 2-site analysis with the equilibrium above is more complex and depends on whether coupling of receptors to G-protein is agonist-dependent or agonist-independent. When coupling is completely agonist-independent, $K_H = K_3$. When coupling is agonist-dependent, K_H is approximated by $K_2.K_3$ because formation of the ternary complex in this case depends on the energetically unfavorable coupling of receptor to G-protein (represented by K_2) and the energetically favorable binding of agonist to the coupled receptor (represented by K_3). For a system with a given K_2, a greater K_L/K_H indicates that the preference of binding to coupled receptors is higher than when K_L/K_H is smaller.

For any given membrane preparation, the relationship between K_L/K_H and R_H depends on whether the coupling of receptor to G-protein is agonist-dependent or agonist-independent. We present both extremes here to define the upper and lower limits of R_H that the TCM allows, in theory, for an agonist with a given K_L/K_H. When coupling is agonist-independent, the magnitude of R_H is predetermined by the extent of constitutive coupling (precoupling). Therefore, a higher K_L/K_H should be reflected in a higher preference of the agonist for RG than for R, but not in an increase in R_H. We should keep in mind here that absolute agonist independence is unlikely because it does not leave space for agonism, but it is assumed here in order to draw the upper limit.

When, on the other hand, the coupling of receptor to G-protein is agonist-dependent, a higher K_L/K_H should be reflected not only in more preference for RG, but also in an increase in receptor coupling to G-protein (higher R_H). In conclusion, the TCM necessitates that R_H values for different agonists at the same receptor system should be either the same or higher for the agonist that has a higher K_L/K_H in a predictable manner depending on the extent of constitutive coupling of receptor to G-protein.

In the Results section, we show experimental R_H – log K_L/K_H relationships along with an upper line and a lower curve representing, respectively, the extremes of agonist-dependent and agonist-independent coupling. For the TCM to be valid, experimental R_Hs should form a smooth curve between these two extremes at a position that depends on the level of agonist-independent precoupling (the value of K_2).

To draw the upper and lower limits of R_H throughout this project, we followed several steps. First, we assumed a 100% and ~0% precoupling for upper and lower limits of R_H, respectively. Drawing the lower limit is laborious; ~0% precoupling was simulated by first assuming a very large value for the equilibrium constant of precoupling (ie, $K_2 = 1000$). We assumed that the ratio of G-protein to receptor is equal to 110% of the R_H of the experimental agonist with the highest R_H value. We also assumed an equilibrium constant for binding of agonist to uncoupled receptor (K_1 in the equilibrium above) of 1×10^{-6} M. This value is arbitrary, but selected to be at a fairly typical value for low affinity binding of an agonist. We then constructed a spreadsheet in which we put equations to solve for [ARG] and [AR] based on K_1, K_2, and K_3 values, theoretical agonist concentrations, and the assumed concentration of G-protein (derivations of equations are presented in Supplement 1). K_3 was varied to obtain a range of log K_L/K_H values. From [ARG] and [AR], the spreadsheet was used to calculate fractions of sites available for antagonist at different agonist concentrations. When these fractions were plotted against the logarithm of agonist concentration, a 2-site curve was obtained. From the analysis of this curve (by Graph Pad software), R_H was obtained for each log K_L/K_H. R_H was then plotted against log K_L/K_H and the relationship was taken as the theoretical lower limit of R_H.

Drawing the upper limit of R_H is straightforward. It is approximated by the plateau, with incremental log K_L/K_H values, of R_H values simulated for the extreme of no precoupling.

In Figure 1 we simulate the impact the degree of precoupling has, in the context of the TCM, on R_H values for theoretical agonists with different log K_L/K_H values. R_H in this figure is obtained from quantitative derivations based on the TCM (Supplement 1) combined with 2-site fit. Assuming that we know the R_H value (say 0.65) for an agonist (A) with a defined log K_L/K_H (say 3.2) in certain experimental conditions, and want to know what the TCM predicts R_H to be for an agonist (B) that has a lower log K_L/K_H (say 0.7). As can be seen in Figure 1, the TCM does not predict a certain exact value, but a range of values for R_H (in this case ranging from 0.4 to 0.65), depending on the extent of precoupling of the
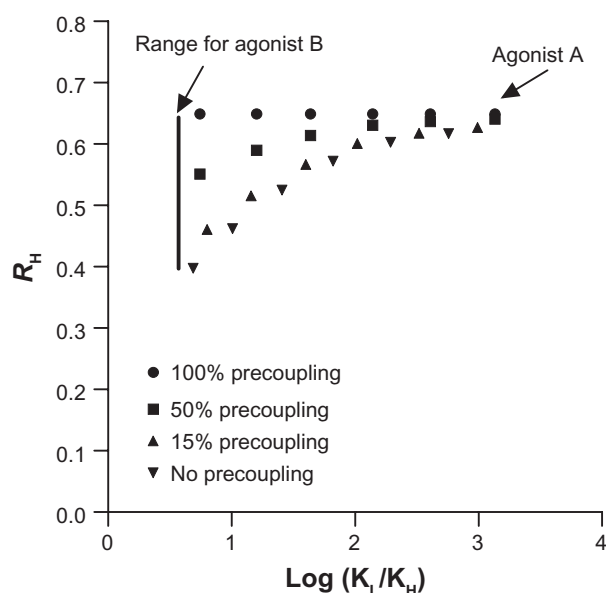
Figure 1 A simulation for the effect of constitutive coupling (precoupling) of receptor to G-protein on the relationship between R_H and log K_L/K_H. K_L and K_H, low and high affinity constants of receptor for agonist, respectively; R_H, fraction of receptors in the high affinity state at saturating agonist concentrations. G/R ratio was fixed at 0.65.

receptors. This argument can be extended to any number of agonists, keeping in mind that the degree of receptor precoupling should have the same impact on all agonists regardless of its magnitude. Therefore, R_H versus log K_L/K_H relationship should form a smooth curve which falls between the two limits of 0% and 100% precoupling.

From this discussion, we can see that the TCM predicts definite relationships between the binding parameters of an agonist (R_H and K_L/K_H). If these relationships are shown not to be valid, the TCM is then insufficient.

Here we have two important notes. First, it may be argued that obtaining data inconsistent with our simulations invalidates a 'restricted version' of the TCM which included the arbitrary values denoted for G/R ratio, K_1, and K_2 in our simulations, so choosing a different set of equilibrium values would modify the lower limit curve and accommodate the data. In response, no matter how these values are manipulated, upper and lower limits will be obtained within which the R_H versus log K_L/K_H relationship should form a curve if the TCM is sufficient. Second, upper and lower relationship limits are also imposed by all TCM extensions and modifications which limit the interaction to a 'ternary' complex; ie, do not incorporate the concept of other players that modify receptor affinity for ligand. For example, in the modification that assumes ligand-specific conformations, the term for K_L is:

$K_L = K_a \times f_{RaA} + K_b \times f_{RbA}$ … where K_a is the affinity constant for conformation a, and f_{RaA} is the fraction of this

conformation of all low-affinity conformations at saturating agonist concentrations. It follows that affinity and fraction terms are still related and the microscopic K_L's, K_H's, and R_H's for individual conformations are all reflected in the macroscopic K_L's, K_H's, and R_H's obtained from 2-site analysis.

In our simulations, we assume that R_H is equal to fRGA (fraction of RGA at saturation agonist concentrations). However, we should mention here that the extent of identity between R_H and fRGA depends on whether coupling of receptor to G-protein is agonist-dependent or agonist-independent. In brief, the divergence between R_H and fRGA increases with increased agonist dependence of receptor coupling to G-protein. This is due to the divergence in these conditions between equation 18 and the equation built in the Graph Pad software to obtain R_H:

$$\left(\frac{Max_{high}[A]}{K_H + [A]} + \frac{Max_{low}[A]}{K_L + [A]} \right)$$

where Max_{high} and Max_{low} are, respectively, the concentrations of receptors bound to the agonist with high and low affinities at saturating agonist concentrations.

Figure 2 is a simulation for the divergence between R_H and fRGA in the extreme of agonist-dependent coupling (a very large K_2 value of 1000). The G-protein/receptor ratio had to be <1.0 to conform to the assumption of the TCM of limited G-protein. We assumed a fixed K_1 value and then varied the value of K_4. At each K_4, we calculated the amount of agonist that should bind to the receptor (to form RA and RGA species) at incremental agonist concentrations and followed the same
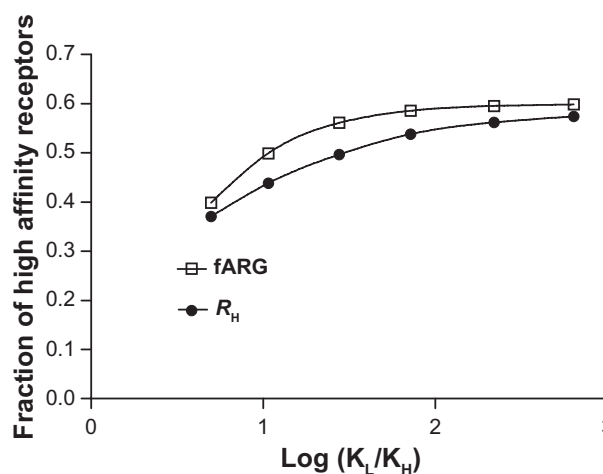
Figure 2 A simulation for the divergence between the fraction of high affinity receptors calculated from equations describing the TCM and that obtained from two-site fit in the extreme of agonist-dependent coupling. The values of K_1, K_2, r, and g were assumed at 10^{-6}, 1000, 1, and 0.6, respectively.

steps as above to get K_L/K_H and $\boldsymbol{R}_H$ values at that given K_4. fRGA was simply taken to be the calculated fraction of RGA species (from the sum of RA and RGA) at saturating agonist concentrations and is given by the equation:

$$fRGA = \frac{r + g + K_4 - \sqrt{(r + g + K_4)^2 - 4rg}}{2}$$

The same process was repeated for different K_4 values. The fraction of receptors with high affinity (calculated fRGA and $\boldsymbol{R}_H$ obtained from Graph Pad analysis) was then plotted against log K_L/K_H.

An important note needs to be made here. Although fRGA and $\boldsymbol{R}_H$ in the simulation above are not identical, they are still proportional. Also, the magnitude of divergence shown in the simulation is at maximum because the extreme of no precoupling is assumed. Therefore, $\boldsymbol{R}_H$ should be considered a useful approximation of fRGA.

Analysis of binding data in the literature

To evaluate whether the relationships between $\boldsymbol{R}_H$ and log K_L/K_H in the literature are consistent with the predictions of the TCM, we analyzed data from studies on a variety of GPCRs including α_2-adrenergic,[20] β-adrenergic,[2,4] muscarinic,[5,11] D_2-dopamine,[10,12,16,34] A_1-adenosine,[3] and 5-HT$_{1A}$ serotonergic[28] receptors. We should mention that in none of these studies was the fact that the TCM imposes certain limits for the relationship between $\boldsymbol{R}_H$ and log K_L/K_H discussed, nor was such a relationship suggested for establishment. In only one instance, a note was made that the β-AR agonists with higher log K_L/K_H ratios had higher $\boldsymbol{R}_H$s ($P < 0.001$, $R^2 = 0.95$).[4] The authors considered this proportionality to signal the validity of the TCM.

Binding experiments

We performed our own binding experiments because we went one step further, involving introducing a binding parameter that correlates with agonist efficacy (manuscript in preparation). This step needed reliably accurate binding data, as well as choosing the suitable expression of efficacy. In that work, we explain what reservations we have on some efficacy expressions in the literature.

The β_2-AR agonists tested were: epinephrine, isoproterenol, fenoterol, procaterol, albuterol, terbutaline, zinterol, dobutamine, tulobuterol, and ephedrine. The β_2-AR-containing membranes were from S49 mouse lymphoma cells, BEAS.2B human bronchial epithelial cells, or human embryonic kidney cells transfected with hemagglutinin-tagged β_2-ARs (HβARH

cells). HβARH membranes were either reconstituted with α-stimulatory and $\beta\gamma$-subunits of G-protein ($G_{s\alpha}$ and $G_{\beta\gamma}$) or not reconstituted.

The reason why we needed to reconstitute HβARH membranes with H_6-$G_{s\alpha}$ and $G_{\beta\gamma}$ in a set of experiments was the low $\boldsymbol{R}_H$ values obtained with the naïve membranes. As expected, reconstitution did increase $\boldsymbol{R}_H$ (from 28.08% to 58.25% for epinephrine), thus increasing the sensitivity of these membranes for comparison of different agonists. However, we then harvested another HβARH membrane batch in which epinephrine had an $\boldsymbol{R}_H$ of ~50%, high enough to resolve differences, if any, between $\boldsymbol{R}_H$s for different β_2-ARs agonists without need for reconstitution. We call this batch of membranes 'naïve' hereafter in the study. The enhanced $\boldsymbol{R}_H$ values we observed with the second batch could be due to better preparation techniques that preserved coupling of receptors to endogenous G-protein.

The low β_2-AR densities in S49 and BEAS.2B membranes put a limit on the number of agonists that could be tested in these membranes.

Materials

S49 cells were obtained from Dr Henrey Bourne (University of California, San Francisco, CA). BEAS.2B cells, HEK cells transfected with hemagglutinin-tagged β_2-AR, hereafter referred to as HβARH cells, and [125I]-radioactive iodinated cyanopindolol, hereafter referred to as [125I]CYP, were kindly provided by Dr Richard Clarke (University of Texas at Houston, Health Science Center, Houston). Ham's F-12 medium, aprotinin, and beta subtype 2 adrenergic receptors, hereafter referred to as β_2-AR agonists, were obtained from Sigma. Fetal bovine and donor horse sera were from Atlanta Biologicals (Norcross, GA). Penicillin and streptomycin were from Mediatech. Tris Base, GTP, and guanosine-51-O-(3-thio)triphosphate (GTPγS) were from Boehringer-Mannheim Biochemicals. Tritiated dihydroalprenolol, hereafter referred to as [3H]DHA, and [32P]ATP were from Dupont NEN. Multiscreen 96-well plates were from Millipore. [3H]cAMP was from Moravek Biochemicals. The plasmid H6-Gsα pQE60 and the bacteria *E. coli* Bl21-DE3/pREP4 were kindly given to the authors by Dr Carmen Dessauer (University of Texas at Houston, Health Science Center, Houston, TX). Ni-NTA agarose resin was purchased from QIAGEN.

Cell culture and membrane preparation

S49 cells were grown and membranes harvested as described by Krumins et al[35] except that buffer B did not contain trypsin inhibitor or leupeptin.

BEAS.2B cells were grown to confluence in T150 flasks at 37°C in sodium bicarbonate-buffered Ham's F-12 medium supplemented with penicillin, streptomycin, and 10% fetal bovine serum. To split the cells, the medium was removed and the cell monolayer washed with 10 mL phosphate buffer solution, then 2 mL of trypsin solution (trypsin 0.25% and EDTA 0.02%) was added to the cell monolayer for 3–5 minutes. Then 10 ml of fresh medium was added and cells were suspended into the medium. For membrane preparation, the cells were passed into cell culture dishes and grown for at least 48 hours. Then the medium was removed by aspiration and the cell monolayer washed twice with 10 mL cold HE solution (20 mM Hepes, pH8 and 1mM EDTA, pH 7.0). The cells were then scraped into 6 mL HE containing the protease inhibitor leupeptin (10 µg/mL). The cells were then homogenized in a type B Dounce homogenizer. Membranes were obtained from the lysate and stored as with S49 lysate with the exception that centrifugation was for 20 minutes.

HβARH cells were grown and membranes prepared using the exact same procedures for BEAS.2B cells with two modifications; Hepes-buffered DMEM was used instead of Ham's F-12 medium, and G418 sulfate was included in the growth medium to select for the cells that were stably transformed with hemagglutinin-tagged β_2-ARs.

Preparation of H_6-$G_{s\alpha}$ and H_6-$G_{\beta\gamma}$

$G_{s\alpha}$ tagged with 6 residues of histidine (H_6-$G_{s\alpha}$) was prepared as described by Lee et al.[36] H_6-$G_{\beta\gamma}$ was prepared as described by Iniguez-Lluhi et al.[37] Protein concentration was determined using the Bradford method.[38]

Reconstitution of HβARH membranes with H_6-$G_{s\alpha}$ and $G_{\beta\gamma}$

To reconstitute HβARH membranes with H_6-$G_{s\alpha}$ and $G_{\beta\gamma}$, a membrane quantity including ~ 0.66 pmole β_2-AR, 1 nmole of each of H_6-$G_{s\alpha}$ and $G_{\beta\gamma}$ and 10 µl 0.01 M $MgCl_2$ were vortexed in a vial for 3 minutes. The vial was then put in ice for 15 minutes to be used for one binding experiment.

Ligand competition

Binding analyses were carried out in 305 µl reactions with a fixed concentration of [125I]CYP or [3H]DHA (75.0 pM and 1.0 nM, respectively), increasing β_2-AR agonist concentrations and an amount of membrane protein containing 8.76 fM of β_2-AR (for S49 and HβARH membranes). The reaction mixture contained the following final concentrations of reagents: 18.0 mM Hepes pH 8.0, 2.2 mM $MgCl_2$, 143.0 µM 1-methyl-3-isobutylxanthine (MIX), 29.0 µM phentolamine, 2.9 µM

ascorbate, 29.0 µM thiourea, and 1.2 mM EDTA pH 7.0. The reaction mixture was incubated for 40 minutes at 30°C. Nonspecific [125I]CYP binding was determined in the presence of 20 µM alprenolol. Not more than 10% of the amount added of [I125]CYP was bound to filter papers, except in case of BEAS.2B membranes where nonspecific binding was up to 35%. Reactions were terminated by vacuum filtration through GF/C filters in 96-well filter plates using Millipore vacuum filtration apparatus and the filters were washed three times with a solution of 10 mM Tris-Cl, pH 7.4 and 5mM $MgCl_2$. The filters were dried and radioactivity counted using a Beckman Gamma counter for 1 minute. For accuracy enhancement, 13–14 agonist concentrations in quintuplets were used. Specifically bound [I125]CYP was plotted against log [agonist] and two-site competition analysis using Graph Pad software version 3.02; Graph Pad, San Diego, CA, yielded values of K_L, K_H, and $\boldsymbol{R}_H$.

Determination of dissociation constants for radioactive antagonists at β_2-AR and β_2-AR density

Similar reaction buffers and conditions were used as in ligand competition assays. Instead of running an agonist-antagonist competition, various concentrations of the radioactive antagonist (~2–500 pM and ~25–2700pM for [125I]CYP and [3H] DHA, respectively) were incubated with membranes in the presence or absence of 20 µM alprenolol. Specifically-bound radioligand concentration was plotted against the logarithm of the free radioligand concentration and a sigmoidal dose-response analysis using Graph Pad software yielded IC_{50} values for the radioligands. Cheng-Prussof correction was used to obtain the overall dissociation constant for binding of ligand to receptor (K_d) values.

To solve for receptor density we first calculated the concentration of radioactive antagonist bound to receptor at saturation. The receptor density was obtained by dividing the moles of receptor by the concentration of membrane protein.

Analysis of data: statistical significance

Relationships were considered significant at $P < 0.05$.

Results
Analysis of binding data in the literature

Our analysis of $\boldsymbol{R}_H$ versus log K_L/K_H relationships is represented in Figure 3 and we obtained the following main results. Only in one study[11] was a relationship within the limits of the TCM obtained. In two other studies[3,4] significant relationships of higher $\boldsymbol{R}_H$ for agonists with higher log K_L/K_H were obtained, but they fell out of the limits of the TCM. In

two other studies[10,12] significant relationships of lower R_H for agonists with higher log K_L/K_H were obtained. In the other seven studies [2a,2b,5,16,20,28,34] no significant relationship between R_H and log K_L/K_H was obtained.

Binding experiments

The relationship between the fraction of β_2-ARs with high affinity for an agonist (R_H) and the ratio between high and low affinities of β_2-ARs for the agonist (log K_L/K_H)

Figure 4 shows typical ligand competition curves obtained for the agonists tested in HβARH membranes against [125I] CYP to determine values of R_H and log K_L/K_H for these agonists.

Figure 5 shows that the experimental R_Hs did not fall within the range predicted by the TCM for a series of β_2-AR agonists that vary in log K_L/K_H in all tested membrane types (S49, BEAS.2B, naïve HβARH, and reconstituted HβARH membranes). Notice, however, that a subset of these agonists (epinephrine, fenoterol, albuterol, terbutaline, and

dobutamine) consistently follows a trend of higher R_H for agonists with higher log K_L/K_H.

Using [3H]DHA as an antagonist, a significant relationship was obtained (Figure 6). However, it is most probable that had the out-players (zinterol, procaterol, and ephedrine) been tested, a result of no significant relationship would have been obtained as with [125I]CYP.

Determination of dissociation constants for radioactive antagonists at β_2-AR and β_2-AR density

Table 1 shows the dissociation constants (K_d) we obtained for [125I]CYP at β_2-AR in different membrane types. K_d For [3H]DHA in naïve HβARH membranes was 55.7 ± 7.1 pM. Also shown in Table 1 are the β_2-AR densities (B_{max}) in all four membrane types.

Discussion

Using a unique approach, in the present study we show the insufficiency of the traditionally-accepted TCM,
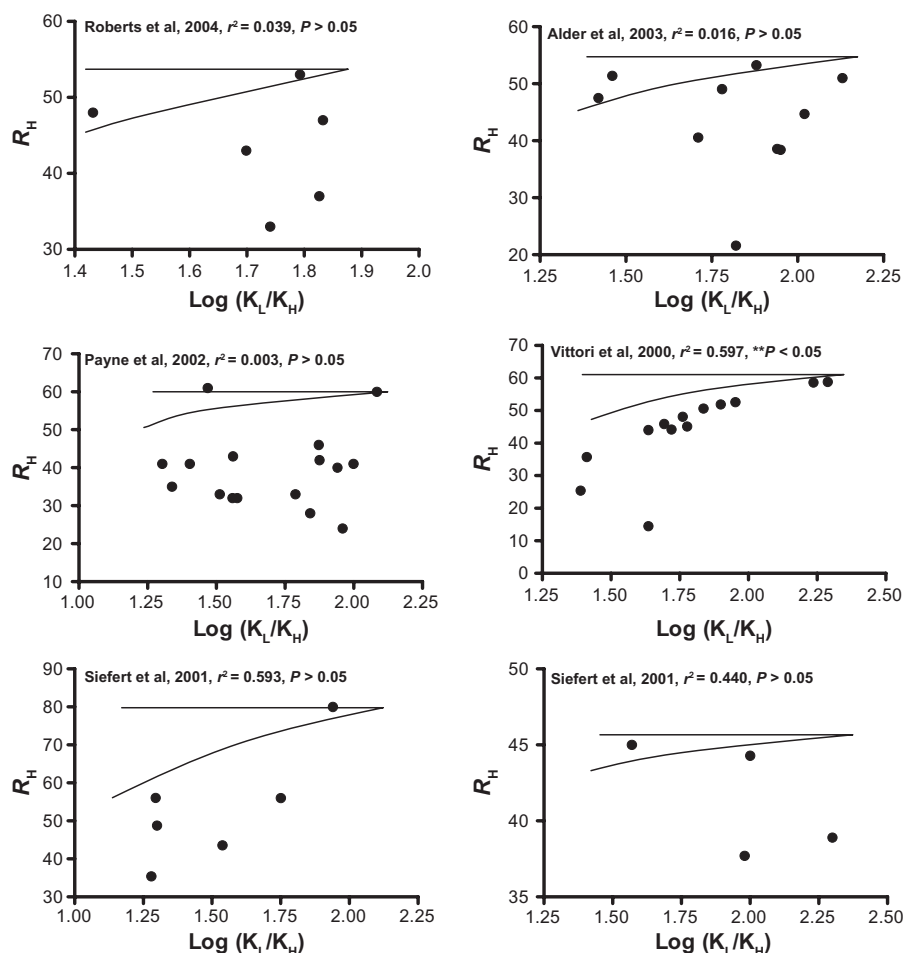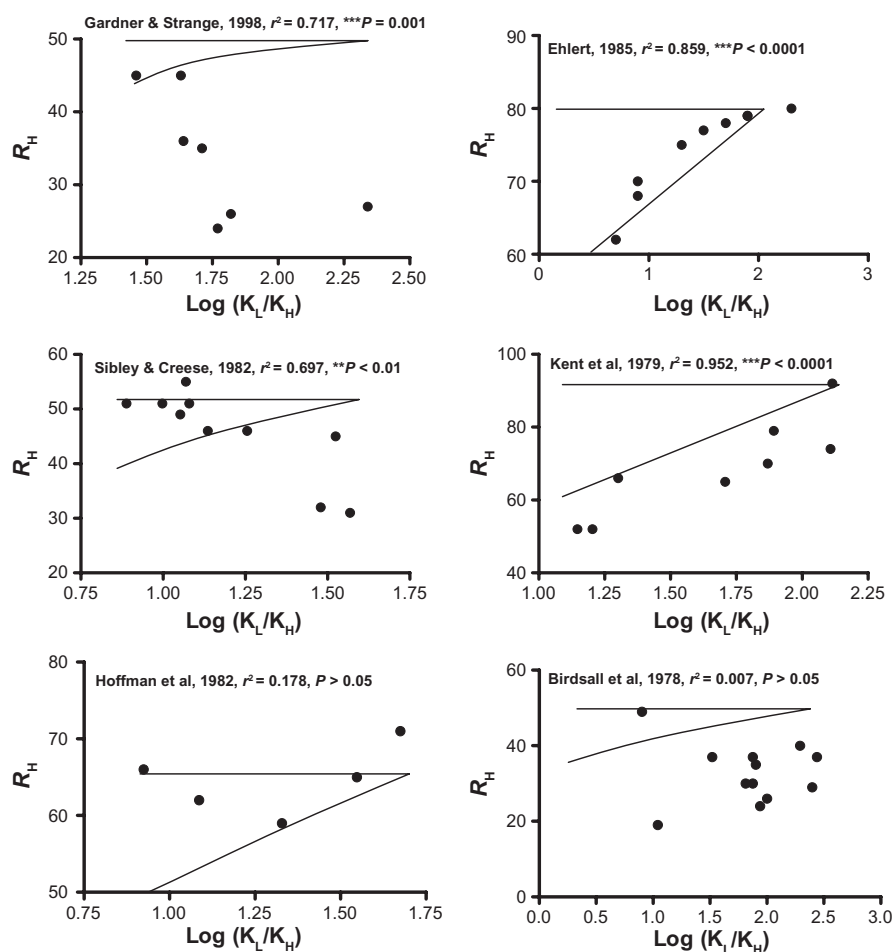

Figure 3 (*Continued*)

Figure 3 Experimental R_H's do not fall within the range predicted by the TCM for a variety of GPCRs in most studies. Upper lines and lower curves represent the R_H's predicted by the TCM in the extremes of agonist-independent and agonist-dependent coupling of receptors to G-protein, respectively. Abbreviations are as in Figure 1.

thus revealing the possible reasons behind inconsistent correlations in the literature between *in vitro* functional efficacy of GPCR agonists and binding parameters derived from the TCM. By careful analysis of binding data in the literature for various GPCRs, we have shown that the experimental relationships between binding parameters [log K_L/K_H and R_H] deviate from the simulations of the relationships that the TCM necessitates (Figure 3). Our own work obtained the same result for β_2-AR agonists in a variety of membrane types and using two different radioactive antagonists (Figures 5 and 6). It is worth mentioning that having some experimental data that follow the predictions of the TCM does not validate the model in this case, since obtaining data inconsistent with an equilibrium model even once indicates its insufficiency.

The novelty of our approach lies in testing relationships between binding parameters (log K_L/K_H and R_H). The stringency imposed by the TCM of these relationships allowed us to reveal discrepancies that could not be revealed by the

much less stringent binding-functional correlations. In other words, even the datasets in the literature that were thought to be consistent with the TCM (based on binding-functional correlations) are generally not so (based on binding-binding relationships).

Thermodynamically, the only way to explain the complex log K_L/K_H versus R_H relationships shown in this study is to recognize that the ternary complex is not simply 'ternary'; ie, other players are included that allosterically modulate receptor affinity. A considerable amount of evidence is cumulating for allosteric modulation of affinity in GPCRs.[28,39–46] In a review article different models that incorporated allosterism into the ternary complex model were discussed and its impact on ligand affinity was described. However, the authors finally pointed implicitly to a conclusion similar ours of discrediting the TCM after presenting experimental observations where 'accessory molecules' or 'coupling partners' modulated the affinities of receptors for agonists.[41] The authors concluded: "Some other additions to the list of GPCR coupling partners
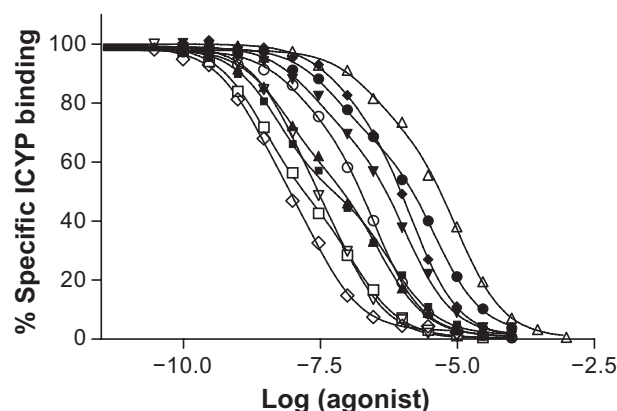
Figure 4 Typical ligand competition curves obtained for the agonists tested in naïve HβARH membranes against [^{125}I]CYP. Each curve is representative of an average of 6 experiments. (▽) procaterol, (◇) zinterol, (○) tulobuterol, (♦) dobutamine, (□) isoproterenol, (■) epinephrine, (▲) fenoterol, (▼) albuterol, (●) terbutaline, (△) ephedrine.

promise to quash the concept of the receptor-G protein signaling hierarchy altogether".

So, our study is not the first to bring to focus the effect of allosterism on ligand affinity in GPCRs. However, we have two very significant additions to what have been discovered so far about allosteric modulators. First, we show here that allosteric modulation is concluded from analysis of simple binding experiments. This is in contrast with the above-mentioned citations which involved evaluations of the

effect of 'adding' an allosteric modulator on receptor affinity for an agonist. Second, our analysis here shows that allosteric modulators exist naturally or in experimental environments for most GPCRs in most systems because, to our knowledge, their presence is the only way to explain complex log K_L/K_H versus R_H relationships (see below). This is in contrast with the citations above which show that a given molecule or ion in a given concentration 'can' modulate binding properties, but whether this modulation does exist naturally remains to be proven.

Now, to appreciate the importance of incorporating allosteric modulation in explaining binding properties, we have to show how it accommodates binding data not accommodated by the TCM and its extensions. As we can see from Figures 3, 5 and 6, the inconsistency of experimental data with the TCM can be summarized in that log K_L/K_H does not predict R_H. This inconsistency is stylized in the simplest form in Figure 7, in which two agonists with the same log K_L/K_H can have different R_H values, inconsistently with the TCM, which necessitates that such agonists should have the same R_H.

Several assumptions have to be made to enable a model of allosteric modulation explaining Figure 7. These assumptions are substantiated by many observations as detailed below. First, there should be *several* allosteric modulators that associate
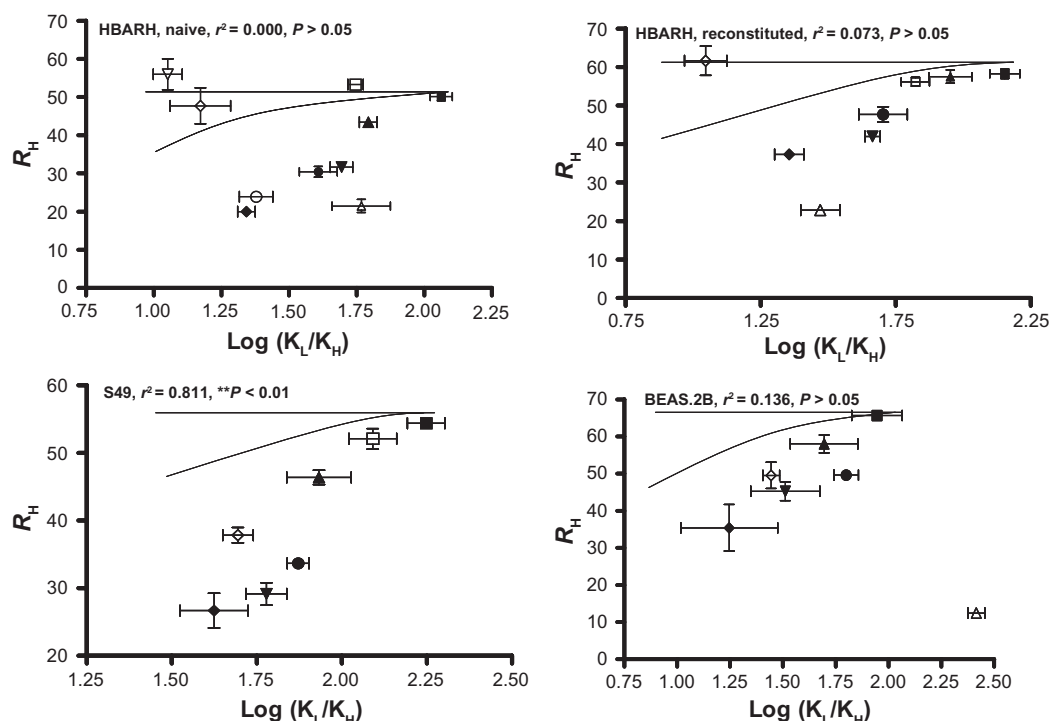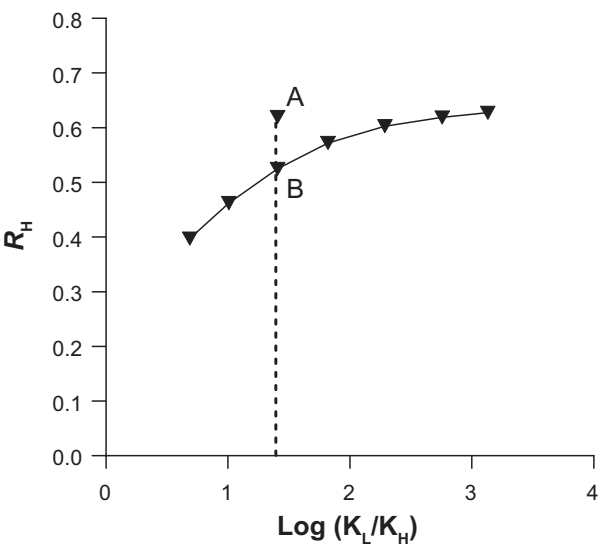

Figure 5 Experimental R_H's do not fall within the range predicted by the TCM for β$_2$-AR agonists in a variety of membranes. [^{125}I]CYP was used as a radioactive antagonist. The upper line and lower curve represent, respectively, the highest and the lowest theoretical R_H's predicted by the TCM. Each point is an average of 6 (naïve HβARH), 9 (reconstituted HβARH), 5 (S49), or 3 experiments (BEAS.2B). Error bars represent SEM's.
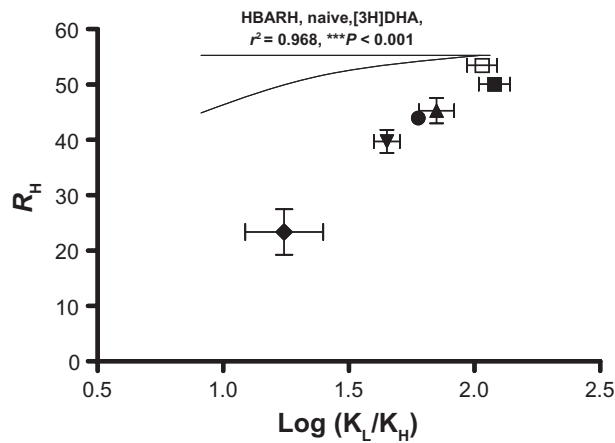
Figure 6 Experimental R_H's do not fall within the range predicted by the TCM for β_2-AR in HβARH membranes. [³H]DHA was used as a radioactive antagonist. Upper line and lower curve represent, respectively, the highest and the lowest theoretical R_Hs predicted by the TCM. Each point is an average of 3–5 experiments. Error bars represent SEM's.

Figure 7 A stylized representation of binding observations that are inconsistent with the TCM. Notice that two agonists (A and B) with the same log K_L/K_H can have different R_H values, which is inconsistent with the TCM.

with a given receptor and modify its affinity for different agonists. Second, different allosteric modulators induce different receptor *conformations*. Third, the induction of a given conformation *changes the difference* between the affinities of two different agonists for the receptor as compared with before the association of the allosteric modulator. In other words, a receptor with an affinity for agonist A that is 10-fold higher than for agonist B before association with the allosteric modulator may have an affinity for A that is, say, 100-fold higher than for B after association with the modulator. Fourth, at least some allosteric modulators are *limited* (lower in concentration than the receptor). Fifth, an allosteric modulator can bind to coupled and/or uncoupled receptors (different observations require different assumptions regarding the receptor species to which the allosteric modulator binds). The 'allosteric modulators model' is the name we give for our model that includes the aforementioned assumptions.

Figure 7 can be explained by the allosteric modulators model as follows. An allosteric modulator (a) associates with the receptor (both coupled and uncoupled forms), increasing its affinity for agonist A more than for agonist B. The affinity of receptor species for A is as follows: R(a)G > R(a) > R. Similarly, an allosteric modulator (b) associates with the receptor (both coupled and uncoupled forms), increasing its affinity for B more than for A. The affinity for B is

R(b)G > R(b) > R. For both agonists, the receptors associated with a modulator are recognized in 2-site analysis as high-affinity sites, whether or not they are coupled to G-protein. Also, (b) is less available than (a), and both are lower in concentration than the receptor. Moreover, we have to assume that the log K_L/K_H intrinsic to B (ie, in absence of (a) and (b)) is higher than the log K_L/K_H intrinsic to A. However, when the two agonists are compared at a receptor system including both (a) and (b), the following happens. The abundance of (a) compensates for the low intrinsic log K_L/K_H of A, thus bringing the log K_L/K_H for A measured in this system to the level of the measured log K_L/K_H for B, which is compromised by the shortage of (b). Also, the abundance of (a) causes the formation of a higher fraction of R(a)G and R(a), as compared with the fraction of R(b)G and R(b) and, therefore, the R_H for A would be higher than the R_H for B.

This type of explanation is the only way by which we have been able so far to account for the complex log K_L/K_H versus R_H relationships obtained in our and others' studies.

The several assumptions made in our model are substantiated by many observations in the literature. For example, agonist-specific conformations have been proposed in several studies.[2,29,30,33]

Table 1 Dissociation constants (K_d) for [¹²⁵I]CYP at β_2-AR and densities (B_{max}'s) of β_2-AR

Membrane type	Naïve HβARH	Reconstituted HβARH	S49	BEAS.2B
K_d for [¹²⁵I]CYP (pM)	26.8 ± 2.0	11.3 ± 0.6	24.8 ± 1.7	15.8 ± 0.6
B_{max} (pmole/mg protein)	4.76	4.40	0.45	0.24

Notes: Data are represented as means ± SEM of two experiments.

As for allosteric modulators that modulate K_L/K_H and/or $\boldsymbol{R}_H$ differently for different agonist, calcyon, a receptor cross-talk protein, reduced $\boldsymbol{R}_H$ for dopamine and SKF82958 at D_1-dopamine receptor without affecting their K_L or K_H values.[40] Importantly, calcyon decreased the difference between $\boldsymbol{R}_H$ values of the two D_1-agonists from 22% to 2%.

In fact, the allosteric modulators do not need to be only newly-recognized entities, but a thermodynamic understanding of molecules already known to interact functionally with GPCR brings to conclusion that they are, in a sense, allosteric modulators. For example, it is well-established that GPCR agonists increase the affinity of GPCR for many molecules, such as PKA and βARK. Thermodynamics requires that any of these molecules should then similarly increase the affinity of the receptor for the agonist. This understanding has been made note of in a recent review of allosteric modulation[45] by mentioning that the expanding list of GPCR-interacting proteins are examples of endogenous allosteric GPCR modulators.

Allosteric modulators need not be only intracellular molecules but can also be ions,[43,44] membrane proteins,[39] or lipids.[45] For example, absence of Ca^{2+} decreased the affinity of GABA for GABAB receptor 25.2-fold while it had no effect on affinity of bacolfen.[44] With dopamine D_2 receptors, replacing Na^+ in the reaction mixture with N-methyl D-glucamine increased log K_L/K_H by 0.21 for the partial agonist aplindore and by 0.35 for dopamine. Yet this replacement increased $\boldsymbol{R}_H$ disproportionately by 37.38% and 7.10% for aplindore and dopamine, respectively.[43] In a third example, a single transmembrane domain protein called RAMP3 (receptor-activity-modifying protein 3) increased the affinity of human calcitonin receptor for rat amylin but not for calcitonin.[39] It can be appreciated now that the recognition of allosteric modulators allows much more flexibility in $\boldsymbol{R}_H$ versus log K_L/K_H relationship than allowed by the TCM and its extensions.

In our experimental settings, the concentration of ions and pH of buffers we used should have influenced the relationship between $\boldsymbol{R}_H$ and log K_L/K_H. Had other concentrations and pH values been used we should have observed different relationships. However, we postulate that an observation will prove consistent no matter what conditions are used; ie, relationships that cannot be explained by the TCM or its extensions.

Our work draws attention to binding-binding relationships as very useful tools for understanding receptor theory and shows that they can be simpler and more definite than the traditionally-applied binding-functional correlations. We did not aim to construct a mass-action scheme for our model

since incorporating allosterism in a model is associated with extreme complexity[41] and is unlikely to have significant practical predictive applicability. On the other hand, the restricted simple forms, such as the allosteric 2-state model, will inevitably fail to explain some allosteric behaviors, as pointed out in a recent review.[45]

Finally, the importance of our work lies in four achievements. First, we invented a novel approach that proved the insufficiency of the ternary complex model and its extensions. Second, this insufficiency of the TCM may account for inconsistency of efficacy-affinity correlations. Third, we provided a model that accounts for binding data inconsistent with the TCM and, at the same time, reveals allosterism intrinsic to various receptor systems. Fourth, our model provides the theoretical basis for deriving a novel binding parameter that correlates with agonist efficacy in our and others' work more significantly and consistently than the parameters used so far (manuscript in preparation).

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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Supplement 1

Here we present our derivation of equations that we used in our spreadsheets to relate the quantities of different receptor species in the context of the TCM:

To calculate R_H predicted by the TCM for an agonist with a given log K_L/K_H we need to calculate the fraction of receptors that is bound to the agonist ([RA] + [RGA]) at different agonist concentrations. The fraction left for binding to radioactive antagonist is then calculated (using a spreadsheet) by subtraction from unity. Then this fraction is plotted against agonist concentration, and R_H as well as log K_L/K_H are obtained using Graph Pad software. So all we need to do mathematically is to solve the equations, based on equilibrium equations and the rule of mass conservation of the TCM, for [RH] and [RGH].

The ternary complex model is described diagrammatically as:

$$K_1$$

$$R \leftrightarrow AR$$

$$K_2 \updownarrow \qquad \updownarrow K_4$$

$$RG \leftrightarrow ARG$$

$$K_3$$
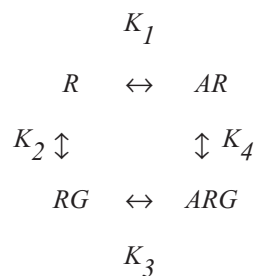
So the equilibrium conditions are:

$$K_1 = \frac{[R][A]}{[RA]} \tag{1}$$

$$K_2 = \frac{[R][G]}{[RG]} \tag{2}$$

$$K_3 = \frac{[RG][A]}{[RGA]} \tag{3}$$

$$K_4 = \frac{[RA][G]}{[RGA]} \tag{4}$$

and the mass conservation conditions are:

$$r = [R] + [RA] + [RG] + [RGA] \tag{5}$$

where r is the total receptor amount

$$g = [G] + [RG] + [RGA] \tag{6}$$

where g is the total G-protein amount

In order to solve for [RGA], we use the equilibrium equations to get expressions for [RA], [RG], and [R] in terms of [RGA] as follows:

$$\text{From 4, } [RA] = \frac{K_4[RGA]}{[G]}: \quad \text{From (3), } [RG] = \frac{K_3[RGA]}{[A]}$$

$$\text{From 1 and 4, } [R] = \frac{K_1[RA]}{[A]} = \frac{K_1 K_4}{[A][G]}[RGA]$$

Insertion of these relationships into equation 5 gives:

$$r = [RGA]\left(\frac{K_1 K_4}{[A][G]} + \frac{K_4}{[G]} + \frac{K_3}{[A]} + 1\right) \tag{7}$$

which may be rewritten as:

$$r = [RGA]\left(\frac{K_4}{[G]}\left(1 + \frac{K_1}{[A]}\right) + 1 + \frac{K_3}{[A]}\right) \tag{8}$$

Now only the elimination of [G] remains. From 6 and 3 we can determine that:

$$[G] = g - [RG] - [RGA] = g - \left(1 + \frac{K_3}{[A]}\right)[RGA] \tag{9}$$

Substituting 9 into 8 gives:

$$r = [RGA]\left(\frac{K_4\left(1 + \frac{K_1}{[A]}\right)}{g - [RGA]\left(1 + \frac{K_3}{[A]}\right)} + 1 + \frac{K_3}{[A]}\right) \tag{10}$$

Writing $(1 + (K_1/[A]))$ as B_1 and $(1 + (K_3/[A]))$ as B_3 gives:

$$r = [RGA]\left(\frac{K_4 B_1}{g - [RGA]B_3} + B_3\right) \tag{11}$$

Multiplying through by g-[RGA]B_3 gives:

$$rg - rB_3[RGA] = K_4 B_1[RGA] + gB_3[RGA] - B_3^2[RGA]^2 \tag{12}$$

Then, rearranging these figure:

$$B_3^2[RGA]^2 - (rB_3 + gB_3 + K_4 B_1)[RGA] + rg = 0$$

Solving using the standard solution to a quadratic gives:

$$[RGA] = \frac{rB_3 + gB_3 + K_4 B_1 - \sqrt{(rB_3 + gB_3 + K_4 B_1)^2 - 4rgB_3^2}}{2B_3^2} \tag{13}$$

This rearranges to:

$$[RGA] = \frac{1}{B_3} \cdot \frac{r + g + K_4\dfrac{B_1}{B_3} - \sqrt{\left(r + g + K_4\dfrac{B_1}{B_3}\right)^2 - 4rg}}{2} \tag{14}$$

In order to calculate the total binding of agonist we also need to know [RA]. We calculate it as follows:

The quantity of receptors not coupled to G-protein (here called x) is equal to [R] + [RA].

From the equilibrium condition (1), [R] is equal to [RA] K1/[A]. Therefore:

$$x = \frac{[RA]K_1}{[A]} + [RA] = [RA]\left(\frac{K_1 + [A]}{[A]}\right)$$

It follows that $[R] + [RA] = [RA]\left(\frac{K_1 + [A]}{[A]}\right)$.

Therefore: $[RA] = \frac{([R]+[RA])[A]}{K_1 + [A]}$

The quantity of receptors not coupled to G-protein ([R] + [RA]) is also equal to r-[RG]-[RGA].

Therefore:

$$[RA] = \left(r - [RGA] - [RG]\right)\frac{[A]}{K_1 + [A]}$$
$$= \left(r - [RGA]\left(1 + \frac{K_3}{[A]}\right)\right)\frac{[A]}{K_1 + [A]} \quad (15)$$

But $1 + (K_3/[A])$ is B_3 and $[A]/(K_1+[A])$ is $1/B_1$. Therefore equation 15 becomes:

$$[RA] = \frac{r - B_3[RGA]}{B_1} \quad (16)$$

By inserting the known solution for [RGA] (equation 14) and rearranging we get:

$$[RA] = \frac{r}{B_1} - \frac{1}{B_1} \cdot \frac{r + g + K_4\frac{B_1}{B_3} - \sqrt{\left(r + g + K_4\frac{B_1}{B_3}\right)^2 - 4rg}}{2} \quad (17)$$

Finally, the total amount of receptor bound by agonist at a given agonist concentration is given by the sum of 14 and 17:

$$[RGA] + [RA] = \frac{r}{B_1} + \left(\frac{1}{B_3} - \frac{1}{B_1}\right).$$

$$\frac{r + g + K_4\frac{B_1}{B_3} - \sqrt{\left(r + g + K_4\frac{B_1}{B_3}\right)^2 - 4rg}}{2} \quad (18)$$