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MOLECULAR PHARMACOLOGY, **52:**144–154 (1997).

The Stability of the Agonist β_2-Adrenergic Receptor-G_s Complex: Evidence for Agonist-Specific States

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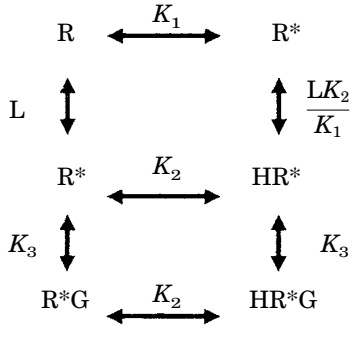
Received December 26, 1996; Accepted April 4, 1997

SUMMARY

A restricted version of the ternary complex model for receptor-G protein complex formation has recently been proposed. Known as the two-state model, this model proposes that in the context of agonist and G protein interactions, only two thermodynamic states exist for the receptor: active (R*) and inactive (R). One form of this model suggests that only the R* state of the receptor is capable of interacting with and subsequently activating G proteins. We directly tested the kinetic aspects of a strict two-state receptor model in a cell line containing the native β_2-adrenergic receptor that is capable of inducing G_s expression. We examined adenylyl cyclase activity in the presence of limiting GTP levels and concluded that there exists a different rate of heterotrimer dissociation (i.e., HR*G yields HR* + G*) for different β_2-agonists. This finding is inconsistent with a strict two-state model in which R* is a characteristic of the receptor that is independent of the identity of the agonist. It implies that agonist activation of adenylyl cyclase is more complicated than a simple two-state model.

The concept of agonist-induced receptor conformation has been the subject of much debate. Although the order of the interaction between agonists, receptors, and G proteins is relatively undisputed, the stoichiometry and nature of the proteins during activation remain very much in question. One model in particular that seems to be gathering widespread acceptance for G protein-coupled receptors is the two-state model for receptor activation (1–3). The two-state model is a restricted version of the ternary complex model (4) and has been extremely useful for explaining the experimental observations involving β_2-adrenergic receptor activation of adenylyl cyclase. Moreover, its stark simplicity makes it easy to set up arguments that attempt to predict the behavior of other features of the system.

In physicochemical terms, the two-state model cannot be exactly true. Molecules exist in a very large number of conformational states, and trying to describe the thermodynamic properties of a receptor with just two conformational states must be an approximation. That given, the usefulness of the model depends on just how reliable its predictions are. In terms of receptor-G protein interactions, a strict two-state model is as shown here:



(1)

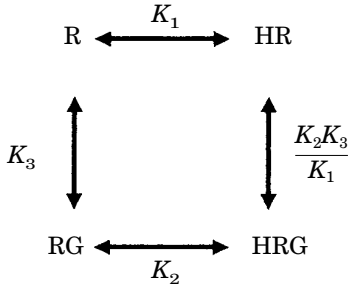
where G is the G protein, R is receptor, and H is agonist. It is supposed that the receptor must exist in one of two conformations (R and R*), with both H and G binding with greater affinity to the R* form. Given this condition, there automatically is cooperativity in binding of the G and H to the R (which explains the GTP shift). Moreover, the different efficiencies of different agonists to promote adenylyl cyclase activation is easily explained by supposing that stronger

This work was supported by National Institutes of Health Grant RR07710.

ABBREVIATIONS: R, inactive form of the β_2-adrenergic receptor; R*, active form of the β_2-adrenergic receptor; HR, agonist-bound receptor; HR*, agonist-activated receptor; RG, preformed receptor-G protein complexes; HR*G, heterotrimeric complex containing activated receptor; HRG_{total}, total heterotrimer complexes comprising all HRG + HR*G species; C, adenylyl cyclase; C_p, proportion of activatable adenylyl cyclase; CYP, iodocyanopindolol.

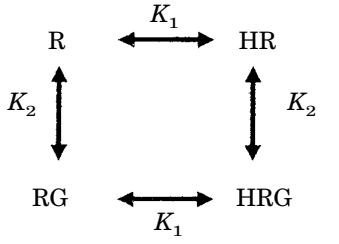
agonists favor binding to the R* form to a greater extent than do weaker agonists and hence more readily form the HR*G ternary complex. In addition, inverse agonists tend to stabilize the inactive R state (5, 6). It must be emphasized that in a strict two-state model, the affinity of G for R is identical to that for HR and its affinity for R* is identical to that for HR* because R and R* represent unique defined conformational states (or collection of states). Given the above set-up, it is possible to develop the thermodynamics of the system in terms of the relative concentrations of the two conformations: when free and when bound to G protein, to agonist or to both.

The classic development of the thermodynamics of the H, R, and G interactions in terms of the ternary complex model (4) shown below is not a one-state but rather a multistate approach. The use of a different dissociation constants for the binding of G to HR than for the binding to R is an acknowledgment that the receptor in HR is in a different conformational state than in R



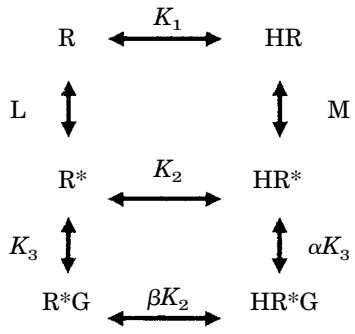
(2)

In this equation, therefore, unlike that for the two-state model, R, HR, and so on represent the totality of possible conformations and not a single conformation or a defined group of conformations in a constant proportion. Thus, although it is superficially simpler than Eq. 1, Eq. 2 is thermodynamically complete and, unlike Eq. 1, it is guaranteed to be correct if the ternary complex model is correct as drawn. Eq. 2 may be compared to a true one-state model shown below in Eq. 3. In this equation, because the receptor can exist in only one conformation, there can be no cooperativity of binding between G protein and agonist.



(3)

The scheme proposed by Samama *et al.* (1) (shown below in Eq. 4), although described in that publication as a two-state scheme, is in fact a multistate model. The fact that different affinities are used for the binding of H to the R* and the R*G state means that a different conformation is assumed for R* when bound and when not bound to G protein. In other words, there are at least three states. The use of this scheme enables one to be completely accurate with the thermodynamics, but it prevents one from making the simplistic two-state arguments, which are the attraction of the model.



(4)

For the two-state model to be useful in successfully making predictions not possible by the classic thermodynamic approach, additional assumptions beyond the schemes discussed above are necessary. If the assumptions are limited to proposing that agonists bind more strongly to the conformation that also more strongly binds G protein, then no argument can be drawn beyond those already possible for the classic thermodynamic scheme. Similarly, negative agonists that reduce the amount of adenylyl cyclase activity below the basal level found in the absence of ligand can be equally well explained (and predicted) by the classic thermodynamic approach. Where the strict two-state model potentially offers advantages is in quantification of predictions and as a potential for a quantitative relationship between thermodynamic properties (e.g., GTP shifts) and kinetic features (e.g., efficiencies of agonists in adenylyl cyclase activation).

In the current study, we investigated the specific hypothesis that the fraction of ternary complex in the activating conformation (i.e., in the HR*G form) determines the rate at which GDP/GTP exchange occurs. In the strict two-state model, the HR*G conformation is the same for all agonists, which differ only in the ratio of HR*G to HRG_{total} [where HRG_{total} = HRG (inactive R) + HR*G (active R)]. The rationale for the test of this hypothesis is to determine the rate of breakdown of the ternary complex for four different β_2 agonists. It will be shown that in all the cases the thermodynamics requires that >85% of the ternary complex must be in the HR*G rather than the HRG form. Proportionality of the rate of breakdown for the ternary complex with the fraction of ternary complex that is HR*G would therefore require that all the rates be similar. This did not occur. We will therefore conclude that the two-state model in its strictest form cannot be applied in all cases.

Experimental Procedures

Materials. Molecular biology reagents, Dulbecco's modified Eagle's medium, and geneticin were obtained from GIBCO BRL (Gaithersburg, MD). Tris Base, GTP, and guanosine-5′-*O*-(3-thio)triphosphate were from Boehringer-Mannheim Biochemicals (Indianapolis, IN). [α-^{32}P]ATP, Na-^{125}I, and [2,8-^{3}H]cAMP were from DuPont-New England Nuclear (Boston, MA). Dexamethasone and the remaining reagents from Sigma Chemical (St. Louis, MO).

Cell culture of an inducible $G_{s\alpha}$ cell line. The establishment and characterization of a stably transfected murine S49 cyc$^-$ T cell lymphoma capable of inducing G_s protein after dexamethasone treatment have been previously described (7). Briefly, the S49 cyc$^-$ cell line, lacking $G_{s\alpha}$ mRNA and protein (8), was electroporated with the

7.7-kb pMMTV · $G_{s\alpha}$ neo vector (a generous gift from J. Gonzales; described in detail in Ref. 9). The vector contains the cDNA encoding rat $G_{s\alpha long}$ linked downstream of the dexamethasone-inducible mouse mammary tumor virus long terminal repeat promoter. The vector also contains the selection marker for neomycin resistance, neomycin phosphotransferase, constitutively driven by the human β-globin promoter.

Transfected drug-resistant cell lines (S49*cyc$^-$) were maintained in stock tissue culture flasks (Corning Glassworks, Corning, NY) at 37° in HEPES-buffered Dulbecco's modified Eagle's medium supplemented with penicillin, streptomycin, 10% heat-treated horse serum, and 200 μg/ml geneticin to maintain selective pressure. Cells were expanded for $G_{s\alpha}$ gene induction experiments into eight individual preconditioned 2-liter roller bottles (Corning) from a single stock source. The final cell density was 1×10^6 cells/ml when the volume was made up to 2 liters with fresh Dulbecco's modified Eagle's medium plus 10% horse serum media. $G_{s\alpha}$ protein induction was initiated when the S49*cyc$^-$ cells were incubated with 5 μM dexamethasone (final, added from a 10 mg/ml stock prepared in 95% ethanol) for times of 1–24 hr. Transfected control cells underwent no treatment.

Membrane preparations. Cell membranes were prepared and isolated as follows. Cells were washed twice with an excess of buffer A (137 mM NaCl, 5.36 mM KCl, 1.1 mM KH_2PO_4, and 1.08 mM Na_2HPO_4, pH 7.2) by centrifugation at $600 \times g$. The cells were then resuspended in ice-cold cell lysis buffer B (20 mM HEPES, 150 mM NaCl, 5 mM NaH_2PO_4, 1 mM EDTA, and 1 mM benzamidine, pH 7.4; buffer B also contained 10 μg/ml trypsin inhibitor and 10 μg/ml leupeptin to protect $G_{s\alpha}$ from possible proteolysis) and placed in a Parr bomb (at 500 p.s.i.) for 25 min. The disrupted cells were centrifuged for 5 min at 600 × g to pellet nuclear debris. The supernatant was layered onto a 23% and 43% sucrose step gradient in HE buffer (20 mM HEPES and 1 mM EDTA, pH 8.0) and centrifuged at 25,000 rpm in a Beckman Instruments (Columbia, MD) SW 28 rotor for 45 min at 4°. The membrane fraction was collected as a band at the sucrose interface. The membranes were immediately frozen in liquid N_2 and stored at −80°. Membrane concentrations were determined using the BioRad (Hercules, CA) assay (10).

β_2 Agonist competition binding: GTP shift analysis. GTP shift binding analyses were used to analyze the total amount of ternary complex (HRG_{total}) present in the S49*cyc$^-$ membrane preparations containing different G_s levels as [GTP] → 0. Binding analyses were carried out in 500-μl reactions with a single 80 pM concentration of the radiolabeled β_2 antagonist ^{125}I-CYP [prepared according to the protocol of Barovsky *et al.* (11) with modifications by Hoyer *et al.* (12)] in the presence of increasing β_2 agonist concentrations and the following final concentrations of the reagents: 1 mM EDTA, pH 7.4, 20 mM HEPES, pH 7.4, 10 μM phentolamine, 0.3 mM $MgCl_2$, and 20–50 μg of cell membranes (diluted with HE, pH 8.0). The binding reaction was conducted in the presence and absence of 10 μM guanosine-5′-*O*-(3-thio)triphosphate for 55 min at 30°. In some experiments, binding was conducted under adenylyl cyclase assay conditions (see below) to examine the effect of varied GTP concentrations on the area of the GTP shift. In all experiments, the nonspecific ^{125}I-CYP binding was determined in the presence of 10 μM alprenolol. Reactions were terminated with the addition of 2.5 ml of ice-cold stop buffer (50 mM Tris-Cl, pH 7.4, 10 mM $MgCl_2$) followed by the immediate filtration of the solution [with the use of a Millipore (Bedford, MA) 1225 vacuum filtration apparatus] onto Whatman (Clifton, NJ) GF/C filters. The reaction tubes were rinsed with an additional 2.5 ml of ice-cold stop buffer. The GF/C filters were washed four or five times with 2.5 ml of stop buffer, dried, and removed to scintillation vials, in which ^{125}I-CYP activity was counted on a Beckman Gamma 4000 System Counter for 1 min. Activity, measured as counts per minute, was converted into units of fmol/mg with the aid of a spread-sheet program using Lotus 1-2-3. The specific binding of ^{125}I-CYP for each concentration was determined as mean values of triplicate measurements for total binding less the mean values of triplicate measurements for nonspecific binding. Data were plotted as log [agonist] versus the normalized fraction of agonist bound and were subsequently analyzed using our recently developed Scatchard method (15) to generate agonist affinity constants (to R and RG, respectively) and $[G_s]_{total}$ and HRG_{total} levels. The use of two-component nonlinear regression analyses (GraphPAD) to analyze the −GTP curve for HRG_{total} levels resulted in values similar to those obtained according to the Scatchard method (±2.5%). The area for the GTP shifts were determined by the trapezoid method. Comparisons of data ± standard deviations were performed using single-factor analysis of variance ($p < 0.05$).

Adenylyl cyclase assays. Adenylyl cyclase assays, conducted with saturating agonist levels and decreasing GTP concentrations, were used to compare the rate of the ternary complex breakdown for different agonists. The premise for using these assays is described in greater detail below and in Discussion. Briefly, it is known experimentally that at normal (high) GTP concentration, the formation of the heterotrimer complex (HRG_{total}) is rate limiting, and once formed, it interacts with GTP extremely rapidly (13). For a strict two-state model, the rate at which the HR*G complex dissociates should be independent of the agonist used to stabilize the R* conformation; therefore, the rate of HRG_{total} breakdown should be proportional to the fraction of HR*G formed by each agonist. The rate of HR*G dissociation was made rate limiting by reducing GTP and allowing the HRG_{total} complex to accumulate.

Adenylyl cyclase activity was examined in S49*cyc$^-$ membranes containing different G_s levels using the conditions for adenylyl cyclase assays as described by Clark *et al.* (14), with some modifications. Briefly, adenylyl cyclase activity in plasma membranes (~0.2 μg/μl, final) was brought to steady state for 3 min at 30° in a 1800-μl volume in a 15 × 100 mm borosilicate glass tube with the following reagents (given as final concentrations): 40 mM HEPES, pH 7.7, 1 mM EDTA, 0.3 mM $MgCl_2$, 8 mM creatine phosphate, 16 units/ml creatine phosphokinase, 0.05 mM ATP, and 0.1 mM 3-isobutyl-1-methylxanthine. The preincubation period was necessary to bring the HRG_{total} levels for each agonist to equilibrium. [For any agonist to be effective, the $t_{1/2}$ of HRG_{total} formation cannot be less than the $t_{1/2}$ for adenylyl cyclase inactivation ($t_{1/2}$ = 15 sec for k_{-1} in S49 cells (16)]. Each assay tube consisted of a different GTP concentration (0, 10, 30, 100, and 300 nM, and 1, 3, and 10 μM), so that for a single agonist, a series of eight independent time courses was required to produce a single GTP dose-response curve. The final concentrations of agonist required to saturate the β_2-adrenergic receptors (i.e., occupy >90% of receptor sites with agonist) were 10 μM for epinephrine (K_d = 1100 nM), 2.2 μM for isoproterenol (K_d = 188 nM), 2.8 μM for fenoterol (K_d = 400 nM), and 13 μM for dobutamine (K_d = 1400 nM).

Time course assays, spanning a 3-min period, were initiated with the addition of 40×10^6 cpm [α-^{32}P]ATP in a 200-μl aliquot containing the concentration of reagents described above. Time course assays were selected over a typical 10-min adenylyl cyclase assay to examine the linearity of the response at low GTP concentrations. The reactions were quenched every 18 sec by removing 100-μl aliquots into 500 μl of ice-cold stop buffer. The isolation and determination of [^{32}P]cAMP activity were performed as described by Salomon *et al.* (15). GTP-dependent adenylyl cyclase dose-response curves were produced using linear regression analysis (GraphPAD, San Diego, CA) to generate the slopes (i.e., cyclase activity) from the linear cAMP accumulation versus time plots. These data were subsequently transformed to Eadie-Hofstee plots to examine the rate of HRG_{total} breakdown (see below). All comparisons of data were performed using single-factor analysis of variance ($p < 0.05$, Excel).

Calculating the limiting HRG_{total} and minimum HR*G levels. The limiting levels of HRG_{total}, as [GTP] → 0, were calculated from the G_s:R ratios obtained from GTP shift analyses for each β_2 agonist. The G_s:R ratios, representing the maximum level of receptor-G protein interaction in the presence of saturating agonist, were obtained for each S49*cyc$^-$ membrane preparation containing variable G_s levels using a newly developed Scatchard method (16, and

data not shown); however, analysis of HR versus HRG_{total} plots for the −GTP curves with a rectangular hyperbola will yield similar results (±2.5%). With the G_s:R level for an agonist, one can determine the limiting HRG_{total} levels by using the following relationship (16):

$$K_{RG} = \frac{[HR]([G]_{total} - [HRG]_{total})}{[HRG]_{total}} \quad (5)$$

where K_{RG} represents the dissociation constant between HR and G. The values used for K_{RG} (unitless when $[R]_{total}$ is set to unity and all concentrations are expressed as fractions or multiples of $[R]_{total}$) were 0.007 ± 0.002 for epinephrine, 0.011 ± 0.006 for isoproterenol, 0.007 ± 0.003 for fenoterol, and 0.029 ± 0.016 for dobutamine, and $[G_s]_{total}$:R ranged from 0.246 to 0.692 when epinephrine was used in GTP shift studies (16). Under saturating agonist conditions, $[HR] \cong [R]_{total}$, and the limiting values for HRG_{total} are determined by solving the following quadratic equation:

$$[HRG]_{total} = \frac{[R]_{total} + [G]_{total} + K_{RG} - \sqrt{([R]_{total} + [G]_{total} + K_{RG})^2 - 4[R]_{total}[G]_{total}}}{2} \quad (6)$$

The minimum HR*G levels are calculated from the HRG_{total} levels using the argument developed in Appendix II. The values for K_{d1}, representing the low agonist affinity binding component, and K_{d2}, representing the high agonist affinity binding component, were generated using a two-component nonlinear regression curve analysis of the −GTP curve for each agonist (GraphPAD). The data representing the limiting HRG_{total} and minimum HR*G values for each agonist were normalized with respect to the HRG_{total} and HR*G levels obtained for epinephrine in the same membrane preparations. The data for different membrane preparations were averaged and analyzed using single-factor analysis of variance ($p < 0.05$, Excel).

Determination of the rate of HRG_{total} breakdown. In a strict two-state model, the rate of HRG_{total} breakdown should be proportional to the fraction of HR*G formed by each agonist (i.e., once HR*G is formed the rate of breakdown should be agonist independent). The experimental rate of HRG_{total} breakdown was determined by fitting the Eadie-Hofstee plots (with reversed axes so that the x-axis represented v and the y-axis represented v/s) for cyclase activity to a quartic solution that kinetically describes the GTP-dependent activation of adenylyl cyclase (Appendix I). A formal derivation reveals that the y-axis in the present case is

$$\frac{k_2[HRG]_{total}C_p}{k_{-1}} \quad (7)$$

where k_{-1} is the rate of G_s^* and G_s^*C inactivation, $[HRG]_{total}$ is the limiting HRG_{total} concentrations, and C_p is the proportion of activatable adenylyl cyclase that is dependent on the G_s level. As [GTP] → 0, [C] = $[C]_{total}$, and is the same for all, and the HR + G → HRG_{total} step is always fast compared with HRG_{total} breakdown; thus, the normal rate of activation for various agonists does not matter. At the y-axis, [GTP] = 0, and the intercept of the line with the y-axis is given by:

$$\frac{k_2[HRG]_{total}C_p}{k_{-1}}, \quad \text{where} \quad \frac{[HRG]_{total}C_p}{k_{-1}} \quad (8)$$

is the same for all agonists. Therefore, the intercepts on the y-axis for the different agonists are in direct proportion to k_2. The data representing k_2 values for each agonist were normalized with respect to the k_2 values obtained for epinephrine in the same membrane preparations. As with the HRG_{total} and HR*G data, the differences in k_2 values were averaged and analyzed using a single-factor analysis of variance ($p < 0.05$, Excel).

Results

Examination of adenylyl cyclase response with varying GTP concentrations. The kinetics of adenylyl cyclase activity, under reduced GTP concentrations, were examined in S49*cyc$^-$ membranes containing variable G_s protein levels as described in Experimental Procedures. At low GTP levels, the system allows heterotrimeric (HRG_{total}) complexes to accumulate, which results in a change in the rate-limiting step of adenylyl cyclase activation from the rate of heterotrimer formation to the rate of active heterotrimer dissociation. The build-up of heterotrimer complexes at low GTP concentrations was confirmed by conducting GTP shift binding analyses with subsaturating GTP levels (4) using adenylyl cyclase assay conditions and demonstrating that the area of the GTP shift was proportional to the level of GTP in the assay (data not shown).

Fig. 1 compares the GTP dependence for adenylyl cyclase activity in the presence of saturating concentrations of four different β_2 agonists. In the absence of GTP, there was a small but detectable adenylyl cyclase activity, which increased from 1.4 ± 0.4 pmol/min/mg for control membranes with minimal G_s expression to 6.73 ± 2.2 pmol/min/mg at high G_s levels. This increase in spontaneous activity presumably reflects the increased G_s levels and the presence of endogenous guanine nucleotides associated with the membrane preparation. Under saturating agonist conditions, the presence of GTP resulted in increased response until the response became saturated at ~3–10 μM GTP. Linear regression analysis of the curves indicated that adenylyl cyclase activity was always linear with time ($r > 0.9$), even under low GTP conditions, during the 3-min course of the assay.

The magnitude of the adenylyl cyclase response was dependent on the increasing G_s levels in the membrane and the type of agonist used to stimulate activity. The rank order of efficacy (epinephrine ≥ isoproterenol > fenoterol > dobutamine) can be seen clearly in a comparison of the normalized adenylyl cyclase activity with respect to increasing GTP concentrations (Fig. 2). Fig. 2 was generated by computing the slopes of the individual linear responses shown in Fig. 1 using linear regression analysis for each GTP concentration. Sigmoid nonlinear regression curve analysis of the GTP response curves also revealed some agonist dependency of the EC_{50} value for GTP, but in most cases the EC_{50} value for GTP was ~100–150 nM. The data shown in Fig. 2 were transformed to Eadie-Hofstee plots to analyze the rate of HRG_{total} breakdown (see below).

Determination of the limiting HRG_{total}, HR*G, and rate of HRG breakdown for each agonist. The test of a true two-state model for receptor activation lies in the assumption that the experimental rate of HRG_{total} dissociation should be proportional to the fraction of activated HR*G complexes. This is predicted to be so if the R* conformation is independent of the type of agonist. Fig. 3 compares the relative levels of HRG_{total}, the minimum HR*G, and rate of ternary complex breakdown for different β_2 agonists.

The limiting level of heterotrimer complex (HRG_{total}) formed in the absence of GTP was calculated for each agonist using GTP shift binding assays (Ref. 16 and data not shown) as described in Experimental Procedures. With a value of 1.0 for epinephrine, the normalized HRG_{total} values (± standard error) for isoproterenol, fenoterol, and dobutamine are 0.92 ±

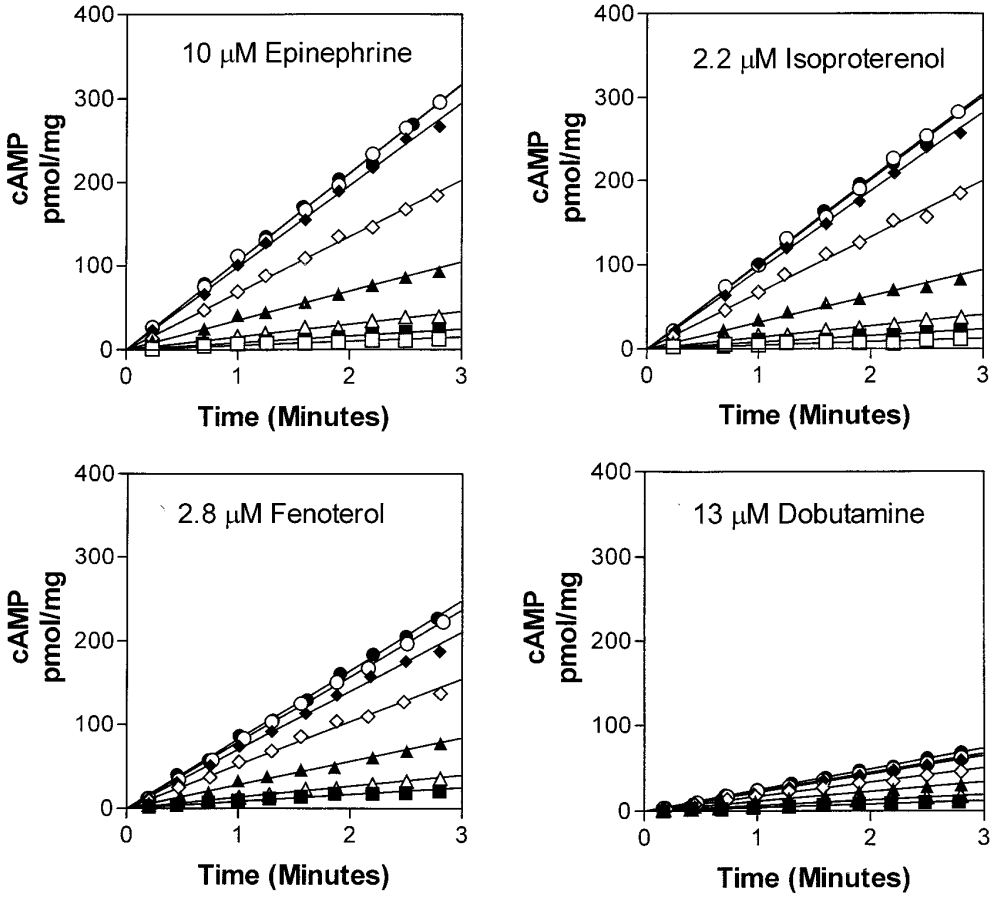


Fig. 1. Comparison of adenylyl cyclase time course studies in S49*cyc$^-$ membranes stimulated with different β_2-agonists. The four plots represent the adenylyl cyclase responses to (A) epinephrine, (B) isoproterenol, (C) fenoterol, and (D) dobutamine for a S49*cyc$^-$ membrane preparation containing the same level of G_s (G_s, $r = 0.7$) in the presence of increasing GTP concentrations (□, 0; ■, 10 nM; △, 30 nM; ▲, 100 nM; ◇, 300 nM; ◆, 1 μM; ○, 3 μM; ●, 10 μM). cAMP accumulation was examined over a 3-min time period in the presence of saturating concentrations of epinephrine (10 μM), isoproterenol (2.2 μM), fenoterol (2.8 μM), and dobutamine (13 μM) as described in Experimental Procedures. Linear regression analysis (GraphPAD) revealed values of $r >$ 0.95 for the curves. These data are representative of the types of responses seen in S49*cyc$^-$ membrane preparations at seven significantly different G_s levels. The eight sets of membrane preparations constitute a series, and three series were performed for epinephrine, whereas the data for isoproterenol, fenoterol, and dobutamine represent measurements in a single series.

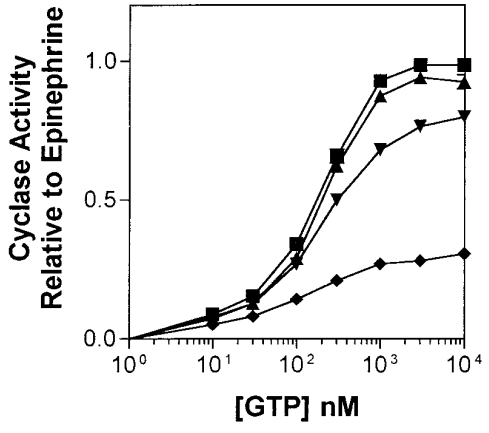


Fig. 2. Comparison of GTP dose response in S49*cyc$^-$ membranes with different β_2-agonists. The GTP dose-response curves for epinephrine (■), isoproterenol (▲), fenoterol (▼), and dobutamine (◆) were generated by calculating the adenylyl cyclase activity from the slopes of each curve in Fig. 1 using linear regression analysis (GraphPAD). The cyclase activity ± standard deviation obtained from linear regression analysis was normalized to a maximum epinephrine elicited activity of 107.7 ± 1.5 pmol/min/mg. The data are demonstrated for a single G_s level ($[G_s]_{total}$, $r = 0.7$) and are representative of the differences between agonist efficacy at seven other G_s levels.

0.04, 0.92 ± 0.04, and 0.82 ± 0.09, respectively. The HRG_{total} calculated levels for isoproterenol, fenoterol, and dobutamine were consistently less than that for epinephrine regardless of the G_s protein level in the plasma membrane (data not shown). An analysis of variance yielded no statistical difference for the effects of G_s protein levels on the measured differences in HRG_{total} levels between the agonists and epinephrine. These data demonstrate that the HRG_{total} levels formed in the absence of GTP seem to be relatively similar from the strong agonist epinephrine to the weak agonist dobutamine.

The values for the minimum HR*G level for each of the agonists were calculated as described in Appendix II and Experimental Procedures. Fig. 3 demonstrates that the minimum fraction of active HR*G heterotrimer complexes makes up a significant proportion (>85%) of the HRG_{total} pool. The HR*G levels for each agonist were normalized relative to the HR*G levels for epinephrine. The relative minimum HR*G values (± standard error) for isoproterenol, fenoterol, and dobutamine are 0.920 ± 0.001, 0.915 ± 0.002, and 0.764 ± 0.006, respectively. The 15% difference between HR*G and HRG_{total} for dobutamine is thought to be due to the difficulty in measuring K_{d2}, the high agonist affinity component; it is highly dependent on the HRG 171 HR*G distribution (in the HRG_{total} pool) in that even a small contribution of HRG (complex with inactive R) leads to a substantial affect on the measured K_{d2}. The use of a two-component nonlinear curve regression analysis to obtain a value for the high affinity binding component (data not shown) revealed that the difference between K_{d1}, the low affinity component, and K_{d2} averaged 28 ± 21-fold for dobutamine (16 experiments), 215 ± 70-fold for fenoterol (16 experiments), 232 ± 82-fold for isoproterenol (eight experiments), and 400 ± 144-fold for epinephrine (24 experiments). The K_{d2} values determined in this fashion also seemed to decrease with increasing G_s levels ($r = -0.94$), which may reflect a complicated receptor-G

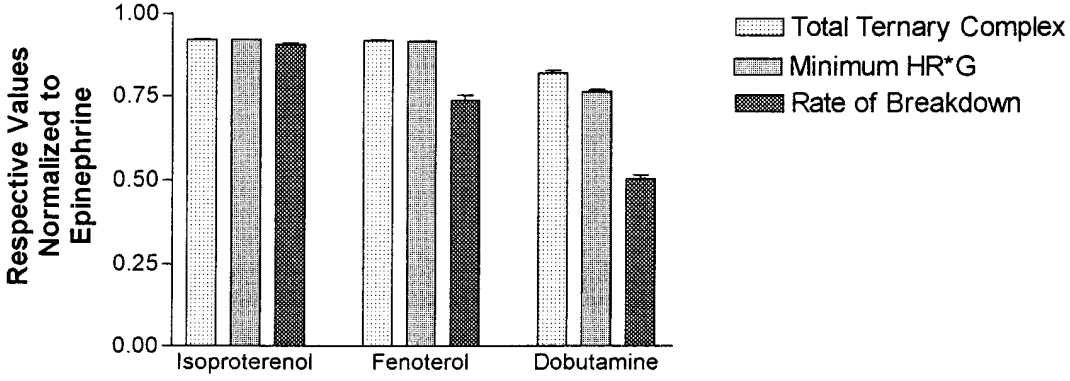


Fig. 3. Comparison of normalized HRG, HR*G, and rate of HRG breakdown for different β_2 agonists. The limiting HRG and HR*G values were then calculated from the G_s:R ratios for each agonist using the method described in Experimental Procedures. The normalized values above represent the average ± standard error from 24 assays for epinephrine, 8 assays for isoproterenol, assays for fenoterol, and assays for dobutamine. With a value of 1.0 for epinephrine, the normalized HRG values for isoproterenol, fenoterol, and dobutamine are 0.92 ± 0.04, 0.92 ± 0.04, and 0.82 ± 0.09, respectively. The values for the minimum HR*G levels were normalized with respect to epinephrine and are 0.906 ± 0.005, 0.915 ± 0.002, and 0.764 ± 0.006 for isoproterenol, fenoterol, and dobutamine, respectively. The rates of breakdown for the HRG complex for each agonist were determined by fitting the quartic solution developed in Appendix I to adenylyl cyclase GTP dose-response data as described in Experimental Procedures. The parameters for $[R]_{total}$ and k_{-1} were arbitrarily set to 1.0. The values for $k_3 = 20$ and $[C]_{total}$:$[R]_{total} = 0.45$ were determined previously using the methods of Krumins and Barber (16). The value for the back-reaction constant k_{10} (0.012) was selected with respect to the k_1 value for dobutamine (0.1) to be consistent with the level of HR*G depicted above. The $[G_s]_{total}$:$[R]_{total}$ values in each S49*cyc$^-$ membrane preparation were dependent on the time of dexamethasone induction and were 0.271, control; 0.378, 1 hr; 0.492, 2 hr; 0.577, 4 hr; 0.659, 6 hr; 0.688, 8 hr; 0.705, 14 hr; and 0.665, 24 hr. At a constant G_s:R level, the rate of breakdown, k_2, served as the only variable when agonist activity was compared. The rate of breakdown data represents the average normalized differences ± standard error between the agonists and epinephrine at each G_s level. The data represent 24 experiments for epinephrine and eight experiments for isoproterenol, fenoterol, and dobutamine.

protein stoichiometry or the difficulty in determining the high agonist affinity using a two-component curve-fitting analysis.

A comparison between the minimum HR*G levels and the relative rate of ternary complex breakdown is also illustrated in Fig. 3. The rate of ternary complex breakdown was determined from the *y*-intercept value of Eadie-Hofstee plots representing GTP-dependent adenylyl cyclase activity for each agonist (see above). The Eadie-Hofstee plots were used to fit a quartic solution describing receptor-G protein/adenylyl cyclase interactions in the presence of decreasing GTP (Appendix I). Using the conditions for fitting as described in Experimental Procedures, the *x*-axis represented the number of active G_s^*-C complexes, a number that is limited by C in this system (16). Therefore in practice, when $G_s \leq C$, the plots were linear; when $G_s \geq C$, the plot tails downward as it approaches the *x*-axis.

In our simulations of fitting the quartic solution to adenylyl cyclase response data (data not shown), only a change in k_2 levels could result in different *y*-intercepts for agonists of different efficacy (i.e., different k_1 values). Using the quartic solution to analyze experimental data revealed that the rates of breakdown (normalized average ± standard error) for isoproterenol, fenoterol, and dobutamine were 0.906 ± 0.005, 0.738 ± 0.015, and 0.505 ± 0.011, respectively. No statistical significance could be demonstrated for the effects of G_s protein levels on the measured differences in the rates of heterotrimer dissociation between the agonists and epinephrine. In addition, no significant difference could be demonstrated between the minimum HR*G levels and the rate of heterotrimer breakdown for either epinephrine or isoproterenol. However, a significant difference could be demonstrated for fenoterol ($p = 4 \times 10^{-3}$, eight experiments) and dobutamine ($p = 1.5 \times 10^{-4}$; eight experiments). These data demonstrate that the rate of breakdown is not similar for different β_2 agonists (even when corrected for disparities in HR*G levels between the agonists), implying that agonist activation of receptors is more complicated than a simple two-state model.

Discussion

Two-state receptor activation model and the rate of HRG_{total} breakdown. The extended ternary complex model has provided an extremely useful thermodynamic description of agonist/receptor-G protein interactions (1–3). This model proposes that the receptor exists in one of two conformations: an inactive state, R, which displays low agonist affinity and presumably does not couple to G (2), and an active state, R*, which displays high agonist affinity and couples to the G protein. The inactive state, R, can isomerize into the active state R*, either spontaneously or with the presence of an agonist. The agonist then stabilizes the R* form, and in general it is accepted that stronger agonists have a greater ability to stabilize the active state.

The two-state model of receptor conformation offers advantages over the more general classic ternary complex model only if it can make verifiable predictions that the latter cannot. There are no qualitative predictions that can be made by the two-state model that are not also obvious from the classic model. The advantage, if there is one, is to use the two-state system to make quantitative predictions based on the amounts of the active form (R*, HR*, or HR*G) present in the system. Essentially, this devolves to relating the quantities (or the relative quantities) of these forms to effector activation or to the steady state of effector activation under conditions in which the nature of the agonist or antagonist is changed.

The scheme for the two-state ternary complex model as illustrated by Samama *et al.* (1), in fact, depicts a multistate model in which $HR^* \xrightarrow{\alpha J} HR^*G$ is dependent on the factor α. If there is complete validity to the strict two-state model as described, $\alpha = 1$. Our data indicate $\alpha \neq 1$. The test for the

two-state model of receptor conformation rested on the following arguments:

1) We give measurements that show the actual accumulation of ternary complex at [GTP] $\rightarrow$ 0 for all the agonists used. In combination with 2) below, we can therefore calculate the maximum differences between the agonists for the steady state concentrations of [HR*G].

2) If the two-state model is an adequate description of the thermodynamics of ternary complex formation, then all of the agonists used are primarily in the HR*G state rather than in the HRG (R inactive) state in the ternary complex (HRG_{total}).

3) We measure the adenylyl cyclase activity for all agonists as [GTP] $\rightarrow$ 0. As [GTP] $\rightarrow$ 0, the rate of breakdown of the ternary complex is the rate-determining step in adenylyl cyclase activation. Measurement of adenylyl cyclase activity under these conditions therefore gives estimates for the relative rates of ternary complex conversion to active G_s^*.

4) The strict two-state model requires proportionality between the amount of HR*G in the system at steady state and the rate of conversion to active G_s^*.

It is known experimentally that at low values of [GTP], the ternary complex accumulates. This is the basis for the theory of the GTP shift for agonist binding (4). In any process involving more than a single step, the intermediate species before the rate-determining step accumulates; therefore, it can reasonably be supposed that when GTP levels are very low, the rate at which adenylyl cyclase is activated becomes directly dependent on the rate at which the ternary complex breaks down. In the current report, however, we performed a complete kinetic analysis over the full range of GTP concentrations from very low to saturating concentrations of GTP. A complete theoretical analysis for the relationship between GTP concentration and adenylyl cyclase activity is given for the shuttle model of cyclase activation (Appendix I). However, although the overall equation is somewhat different when developed for a precoupled model (18), the conclusion that the rate of breakdown of the ternary complex can be inferred from the adenylyl cyclase activity is the same in both cases. It should be pointed out that the exact form of the relationship between adenylyl cyclase activity and GTP concentration is not crucial to the test of the two-state model performed in this report. The solution for that relationship was derived using a G_s-to-C shuttle model as the primary mechanism for adenylyl cyclase activation because it is interesting in its own right and because it allows the mathematical demonstration that as [GTP] $\rightarrow$ 0, the adenylyl cyclase activity is directly proportional to the rate of ternary complex breakdown (Appendix I).

Experimental conditions were chosen such that similar levels of HRG_{total} were used to stimulate adenylyl cyclase activity for four different β_2-adrenergic agonists: the strong agonists epinephrine and isoproterenol, the moderate agonist fenoterol, and the weak agonist dobutamine. If a strict two-state model were manifest for receptor conformation, then similar levels of HR*G are predicted for each agonist when examining adenylyl cyclase activity under limiting GTP concentrations. Appendix II provides the formal thermodynamic justification of the statement above by showing that it is possible to use GTP shift binding data to calculate the minimum percentage of HRG_{total} that must be in the HR*G state. Fig. 3 reveals the similarity in the HR*G levels between the agonists and shows that the weak agonist dobutamine is capable of producing >80% of the HR*G complexes as epinephrine under saturating agonist conditions. Because the percentage is very high even for the weak agonist dobutamine, the subsequent arguments are facilitated.

If the properties of the ternary complex depended proportionately only on the fraction in the active HR*G form, then there should be little difference between the ternary complex that included the weak agonist dobutamine, the stronger agonist fenoterol, and the very strong agonists epinephrine and isoproterenol. It would then be expected that the rate of HRG_{total} breakdown would be similar for all of the agonists. Significant differences in the rates of breakdown would demonstrate that a simple two-state model is inappropriate for this process. Fig. 3 demonstrates that the relative rates of heterotrimer breakdown for fenoterol and dobutamine are significantly different from the minimum HR*G levels determined for each of the two agonists. This suggests that the rate of HRG_{total} breakdown is dependent on the type of agonist, a finding that is inconsistent with a single conformation for the activated state of the receptor.

It is unlikely that the agonist-dependent differences in the rate of heterotrimer breakdown are due to 1) the ability of a particular agonist to form the ternary complex or 2) the agonist-dependent variation in the rates of agonist association and dissociation to the β_2-adrenergic receptor. Our experiments were designed to minimize the effects described above by bringing adenylyl cyclase activity to steady state during a 3-min incubation period before the addition of labeled ATP and subsequent data collection. Therefore, problems resulting from the delay in reaching full activity on the addition of stimulating agonist do not arise. Similarly, the preincubation ensures that agonist binding has also attained a steady state before measurements are taken. Thus, the rates at which agonist binds and dissociates from the receptor should also not be a significant factor.

A possible explanation for these data that would preserve a strict two-state model requires the selective activation of the inhibitory heterotrimeric G protein, G_i. If G_i were preferentially activated by dobutamine and fenoterol, then the reduced adenylyl cyclase activity at low GTP, under stimulation by those agonists, would be the result of G_i-mediated inhibition rather than a slower rate of breakdown of the ternary complex. In the current circumstances, this is unlikely because G_i-coupled α-adrenergic receptors have not been demonstrated in these cells. In addition, the low level of β-adrenergic receptors makes it unlikely that a low-efficiency interaction between the β-adrenergic receptor and G_i could have a significant effect.

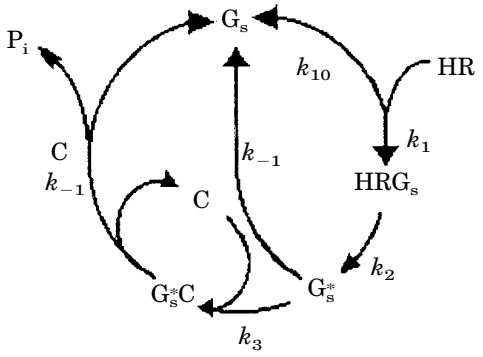
Our data therefore are not consistent with a strict two-state model as described by Samama *et al.* (1). Our data do not, however, invalidate the classic ternary complex model (4), but they do suggest that receptor activation of G_s is more complicated than agonist stabilization of an active R* state. Indeed, other investigators have suggested that each agonist stabilizes a unique set of receptor conformations (known as the multistate model) with a range of G protein-activating abilities (17, 18). However, caution should be taken in extrapolating data from solubilized receptor systems and mutant receptors to biological systems. It should also be noted that although other models for receptor activation are possible, it may be that a more realistic model of agonist/receptor/G protein interactions might only complicate matters

while only marginally improving the resolution of the two-state model. In any case, these findings have important implications not only in the study of the mechanisms involved in hormone-activated receptors but also for future studies involving the search pharmaceutical agents that may selectively activate different forms of a receptor.

Appendix

This section describes the derivation of a complete kinetic analysis of steady state adenylyl cyclase activity with respect to varying GTP concentrations in terms of the Cassel-Selinger cycle (19). The scheme shown below shows the cycle of G_s activation and inactivation after receptor activation. In this version, a shuttle model for G_s^* to adenylyl cyclase catalytic unit is assumed, although an analysis of the process in terms of a precoupled model for G_s and adenylyl cyclase would give almost identical values for the estimates of the rate of breakdown from experimental data. The description of active adenylyl cyclase is given as the solution of a quartic equation; however, the quartic itself can be factored into two quadratic equations, which simplifies matters somewhat.

In the scheme above, HR is the agonist-occupied receptor,



G_s is the inactivated stimulatory G protein, G_s^* is activated G protein, C is inactive adenylyl cyclase, and G_s^*C is the active adenylyl cyclase complex. The kinetic constants governing the cycle are represented by k_1, the rate of heterotrimer (HRG) formation; k_{10}, the rate of dissociation of an inactive heterotrimer; k_2, the rate of active heterotrimer (HRG) dissociation; k_3, the rate of adenylyl cyclase activation by activated G_s^*, and k_{-1}, the rate of G_s^* and G_s^*C inactivation via the intrinsic GTPase mechanism [because adenylyl cyclase does not seem to act as a GAP for G_s^* (20) k_{-1}, the rates for G_s^* and G_s^*C are identical].

In the presence of GTP, we want to determine the steady state rate of adenylyl cyclase, G_s^*C activity. From the conservation of mass, the levels of R, G_s, and C are defined as

$$[G_s]_{total} = [G_s] + [HRG] + [G_s^*] + [G_s^*C] \quad (10)$$

$$[C]_{total} = [C] + [G_s^*C] \quad (11)$$

and under the experimental condition of saturating agonist

$$[R]_{total} = [HR] + [HRG] \quad (12)$$

At steady state, the amount of active G_s^*C in the system is

$$k_3[G_s^*][C] = k_{-1}[G_s^*C] \quad (13)$$

and the amount of active G_s^* in the system is

$$k_1[HR][G_s] = k_{-1}([G_s^*C] + [G_s^*]) + k_{10}[HRG] \quad (14)$$

and the amount of [HRG] is

$$k_1[HR][G_s] = (k_2[GTP] + k_{10})[HRG] \quad (15)$$

By substituting eq. 11 into eq. 13, we obtain

$$[G_s^*] = \frac{k_{-1}[G_s^*C]}{k_3([C]_{total} - [G_s^*C])} \quad (16)$$

Eqs. 12, 14, and 15 can be rewritten as

$$k_1[G_s]([R]_{total} - [HRG]) = k_{-1}([G_s^*C] + [G_s^*]) + k_{10}[HRG] \quad (17)$$

and

$$k_1([R]_{total} - [HRG])[G_s] = (k_2[GTP] + k_{10})[HRG] \quad (18)$$

Therefore,

$$[HRG] = \frac{k_{-1}([G_s^*C] + [G_s^*])}{k_2[GTP]} = \frac{k_{-1}([G_s^*C] + [(k_{-1}[G_s^*C])/(k_3([C]_{total} - [G_s^*C]))])}{k_2[GTP]} \quad (19)$$

and

$$[G_s] = \frac{(k_2[GTP] + k_{10})[HRG]}{k_1([R]_{total} - [HRG])} \quad (20)$$

Substitution of HRG from eq. 19 into eq. 20, after some rearrangement, yields

$$[G_s] = \frac{(k_2[GTP] + k_{10})\left\{\dfrac{k_{-1}\left([G_s^*C] + \dfrac{k_{-1}[G_s^*C]}{k_3([C]_{total} - [G_s^*C])}\right)}{k_2[GTP]}\right\}}{k_1\left\{[R]_{total} - \dfrac{k_{-1}\left([G_s^*C] + \dfrac{k_{-1}[G_s^*C]}{k_3([C]_{total} - [G_s^*C])}\right)}{k_2[GTP]}\right\}} \quad (21)$$

Substitution of eq. 21 for $[G_s]$, eq. 19, for [HRG] and of eq. 16 for $[G_s^*]$ into eq. 10 yields

$$[G_s]_{total} = \frac{k_{-1}\left([G_s^*C] + \left(\dfrac{k_{-1}[G_s^*C]}{k_3([C]_{total} - [G_s^*C])}\right)\right)\left(\dfrac{k_2[GTP] + k_{10}}{k_2[GTP]}\right)}{k_1\left([R]_{total} - \dfrac{k_{-1}\left([G_s^*C] + \dfrac{k_{-1}[G_s^*C]}{k_3([C]_{total} - [G_s^*C])}\right)}{k_2[GTP]}\right)} + \frac{k_{-1}\left([G_s^*C] + \dfrac{k_{-1}[G_s^*C]}{k_3([C]_{total} - [G_s^*C])}\right)}{k_2[GTP]} + \frac{k_{-1}[G_s^*C]}{k_3([C]_{total} - [G_s^*C])} + [G_s^*C] \quad (22)$$

By letting $x = [G_s^*C]$ and $y = ([C]_{total} - [G_s^*C])$, we obtain

$$[G_s]_{total} = \frac{k_{-1}\left(x + \frac{k_{-1}x}{k_3 y}\right)\left(\frac{k_2[GTP] + k_{10}}{k_2[GTP]}\right)}{k_1\left([R]_{total} - \frac{k_{-1}\left(x + \frac{k_{-1}x}{k_3 y}\right)}{k_2[GTP]}\right)} + \frac{k_{-1}\left(x + \frac{k_{-1}x}{k_3 y}\right)}{k_2[GTP]} + \frac{k_{-1}x}{k_3 y} + x \tag{23}$$

Therefore,

$$\begin{aligned} &k_1\left([R]_{total} - \frac{k_{-1}}{k_2[GTP]}\left(x + \frac{k_{-1}x}{k_3 y}\right)\right)[G_s]_{total} \\ &= k_{-1}\left(x + \frac{k_{-1}x}{k_3 y}\right)\left(\frac{k_2[GTP] + k_{10}}{k_2[GTP]}\right) + \frac{k_{-1}}{k_2[GTP]} \\ &\cdot \left(x + \frac{k_{-1}x}{k_3 y}\right)k_1\left([R]_{total} - \frac{k_{-1}}{k_2[GTP]}\left(x + \frac{k_{-1}x}{k_3 y}\right)\right) \\ &+ x\left(1 + \frac{k_{-1}}{k_3 y}\right)k_1\left([R]_{total} - \frac{k_{-1}}{k_2[GTP]}\left(x + \frac{k_{-1}x}{k_3 y}\right)\right) \end{aligned} \tag{24}$$

After some rearrangement and writing

$$\left(1 + \frac{k_{-1}}{k_3 y}\right) \tag{25}$$

as z, we obtain

$$\begin{aligned} &\frac{k_1 k_{-1}}{k_2[GTP]}\left(1 + \frac{k_{-1}}{k_2[GTP]}\right)(xz)^2 \\ &- \left\{k_{-1} + \frac{k_{-1}k_{10}}{k_2[GTP]} + k_1[R]_{total}\left(1 + \frac{k_{-1}}{k_2[GTP]}\right)\right. \\ &\left. + \frac{k_1 k_{-1}}{k_2[GTP]}[G_s]_{total}\right\}(xz) + k_1[R]_{total}[G_s]_{total} = 0 \end{aligned} \tag{26}$$

Eq. 26 is a quadratic in xz and can be solved as a quadratic to give xz, but

$$xz = [G_s^*C]\left(1 + \frac{k_{-1}}{k_3([C]_{total} - [G_s^*C])}\right) = A, \tag{27}$$

where A is the solution to the previous quadratic. Then

$$[G_s^*C](k_3[C]_{total} - k_3[G_s^*C] + k_{-1}) = k_3 A[C]_{total} - k_3 A[G_s^*C]$$

or

$$[G_s^*C]^2 - \left([C]_{total} + \frac{k_{-1}}{k_3} + A\right)[G_s^*C] + A[C]_{total} = 0 \tag{28}$$

Relating the rate of heterotrimer breakdown to the *y*-intercept of the Eadie-Hofstee plot. From eq. 27, we find that

$$xz = [G_s^*C]\left(1 + \frac{k_{-1}}{k_3([C]_{total} - [G_s^*C])}\right). \tag{29}$$

When $[GTP] \to 0$, $[G_s^*C]$ also approaches 0 and

$$xz \to [G_s^*C]\left(1 + \frac{k_{-1}}{k_3[C]_{total}}\right). \tag{30}$$

Substituting this relationship for xz into eq. 26 yields

$$\begin{aligned} &\frac{k_1 k_{-1}}{k_2[GTP]}\left(1 + \frac{k_{-1}}{k_2[GTP]}\right)\left(1 + \frac{k_{-1}}{k_3[C]_{total}}\right)^2[G_s^*C]^2 \\ &- \left\{k_{-1} + \frac{k_{-1}k_{10}}{k_2[GTP]} + k_1[R]_{total}\left(1 + \frac{k_{-1}}{k_2[GTP]}\right)\right. \\ &\left. + \frac{k_1 k_{-1}}{k_2[GTP]}[G_s]_{total}\right\}\left(1 + \frac{k_{-1}}{k_3[C]_{total}}\right)[G_s^*C] \\ &+ k_1[R]_{total}[G_s]_{total} = 0 \end{aligned} \tag{31}$$

Because $[GTP] \to 0$, then

$$1 + \frac{k_{-1}}{k_2[GTP]} \gg 1 \tag{32}$$

in eq. 31, which yields approximately:

$$\begin{aligned} &\frac{k_1 k_{-1}^2}{k_2^2[GTP]^2}\left(1 + \frac{k_{-1}}{k_3[C]_{total}}\right)^2[G_s^*C]^2 \\ &- \left\{\frac{k_{-1}k_{10}}{k_2[GTP]} + \frac{k_1 k_{-1}[R]_{total}}{k_2[GTP]} + \frac{k_1 k_{-1}[G_s]_{total}}{k_2[GTP]}\right\} \\ &\cdot \left(1 + \frac{k_{-1}}{k_3[C]_{total}}\right)[G_s^*C] + k_1[R]_{total}[G_s]_{total} = 0 \end{aligned} \tag{33}$$

Then, writing

$$\theta = \frac{[G_s^*C]}{[GTP]} \tag{34}$$

and

$$\phi = \frac{k_{-1}}{k_2}\left(1 + \frac{k_{-1}}{k_3[C]_{total}}\right)\theta$$

in eq. 33, we obtain

$$k_1\phi^2 - \{(k_1[R]_{total} + k_{10}) + k_1[G_s]_{total}\}\phi + k_1[R]_{total}[G_s]_{total} = 0 \tag{35}$$

The solution to the quadratic eq. shown in eq. 35 is

$$\phi = \frac{k_1([R]_{total} + [G_s]_{total}) + k_{10} \pm \sqrt{\{k_1([R]_{total} + [G_s]_{total}) + k_{10}\}^2 - 4k_1^2[R]_{total}[G_s]_{total}}}{2k_1} \tag{36}$$

Rearrangement yields

$$\phi = \frac{([R]_{total} + [G_s]_{total}) + (k_{10}/k_1) \pm \sqrt{\{([R]_{total} + [G_s]_{total}) + (k_{10}/k_1)\}^2 - 4[R]_{total}[G_s]_{total}}}{2} \tag{37}$$

The right side of eq. 37 is, in fact, the quadratic solution to the formation of HRG_{total}; see Experimental Procedures

where

$$K_{RG} = \frac{k_{10}}{k_1} \tag{38}$$

Therefore,

$$\phi = [HRG]_{total}$$

and replacing ϕ yields

$$\frac{k_{-1}}{k_2}\left(1 + \frac{k_{-1}}{k_3[C]_{total}}\right)\theta = [HRG]_{total} \tag{39}$$

We previously demonstrated for a G_s-to-C shuttle mechanism for adenylyl cyclase activation that $k_3 \gg k_{-1}$ (16). Therefore,

$$\left(1 + \frac{k_{-1}}{k_3[C]_{total}}\right) \rightarrow 1 \tag{40}$$

and because [GTP] $\rightarrow$ 0, eq. 39 simplifies to

$$\theta = \frac{[G_s^*C]C_p}{[GTP]} = \frac{k_2[HRG]_{total}C_p}{k_{-1}} \tag{41}$$

where C_p represents the fraction of adenylyl cyclase activity dependent on the $[G_s]_{total}$ level (i.e., when $[G_s]_{total} < [C]_{total}$, then C_p is proportional to $[G_s]_{total}$, whereas when $[G_s]_{total} > [C]_{total}$, then C_p is proportional to $[C]_{total}$).

Appendix II

The thermodynamics of receptor/G protein/agonist interactions in terms of the two-state model. In this section, we use the two-state model to calculate the minimum fraction of receptors that must be in the R* state in the ternary complex to achieve the measured GTP shifts. If a receptor exists in two distinct conformational states that have different affinities for a ligand (K_a for state A and K_b for state B), then the measured dissociation constant (K_d) for the ligand is given by $K_d = K_{afa} + K_{bfb}$, where fa is the fraction of receptor in state A in the receptor/ligand complex, and fb is the fraction of receptor in state B in the ligand/receptor complex. Because this is a two-state model, fa plus fb is equal to unity. The above equations is derived from the first principles in Appendix III.

If we adhere strictly to the two-state model, then when a ligand binds to a given receptor with increased affinity as a result of a change in conditions, according to the above equation, which is completely general, all changes in K_d must be interpreted in terms of changes in fa and fb, because K_a and K_b refer to the binding to the two defined states.

In the absence of GTP, the β-adrenergic receptor forms a ternary complex with G_s and agonist (4). In this ternary complex, the affinity of the agonist for the receptor is increased relative to its binding in the presence of GTP when ternary complex does not accumulate. If B is the high affinity form of the receptor, then $K_b < K_a$, and the greater measured affinity for ligand in the absence of GTP requires there be a greater contribution of the B form to the ternary complex. In fact, the measured greater affinity places limits on the fraction of each conformation that is thermodynamically allowable.

If K_{d1} is the dissociation constant of ligand from the receptor alone and K_{d2} is the dissociation constant from the ternary complex, then we may write $K_{d1} = K_{afa1} + K_{bfb1}$ and $K_{d2} = K_{afa2} + K_{bfb2}$. Noting that $fa1 = 1 - fb1$ and $fa2 = 1 - fb2$, these two equations may be combined and rearranged to give

$$fb2 = \frac{K_a\left(1 - \frac{K_{d2}}{K_{d1}}\right)}{K_a - K_b} + fb1 \tag{42}$$

The minimum value for $fb2$ occurs when K_b and $fb1$ approach zero. Under these circumstances,

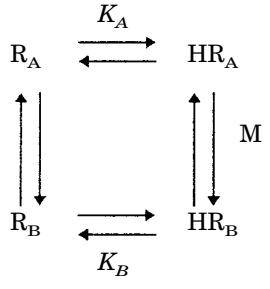
$$fb2 = 1 - \frac{K_{d2}}{K_{d1}} \tag{43}$$

Any other (positive) values for K_b and $fb1$ give a greater value for $fb2$.

It can be seen by inspection that any significant difference in K_{d1} and K_{d2} must lead to large proportion of receptors (>0.9) in the $fb2$ state. In the specific case of this report, even the rather weak agonist dobutamine requires that ≥80% (based on the differences in affinity obtained using a nonlinear two-component curve fit with the high and low values of the curve constant in addition to the low affinity portion of the curve) of the ternary complex be in the HR*G form (i.e., the active form) in terms of the two-state model.

Appendix III

In the following system, only a single dissociation constant is observed provided the conversion time between the different state of the receptor R ↔ R* is rapid compared with the time allowed for equilibration of ligand binding.



(44)

In this scheme, the dissociation for agonist binding K_D is given as

$$K_D = \frac{[\text{Free receptor}][H]}{[\text{Bound receptor}]} = \frac{([R_A] + [R_B])[H]}{([HR_A] + [HR_B])} \tag{45}$$

Because

$$K_A = \frac{[R_A][H]}{[HR_A]}, \quad K_B = \frac{[R_B][H]}{[HR_B]}, \quad \text{and} \quad M = \frac{[HR_A]}{[HR_B]} \tag{46}$$

we can write

$$K_D = \frac{\left(\frac{M[HR_B]K_A}{[H]} + \frac{[HR_B]K_B}{[H]}\right)[H]}{M[HR_B] + [HR_B]} \tag{47}$$

$$= \frac{MK_A + K_B}{M + 1} = f_{HR_A} K_A + f_{HR_B} K_B$$

where f_{HR_A} and f_{HR_B} are, respectively, the fractions of receptor in the A and B conformations *after* ligand has bound.

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