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Inhibition of Adenylate Cyclase Is Mediated by the High Affinity Conformation of the α_2-Adrenergic Receptor

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SUMMARY

The functional significance of high affinity agonist binding to receptors that interact with guanine nucleotide regulatory proteins has remained controversial. Preincubation of human platelet membranes with the full α_2-agonist UK 14,304 in the absence of GTP increases the potency of the agonist to inhibit adenylate cyclase in a pre-steady state (15-sec) assay. The EC_{50} after preincubation (6 ± 1 nM) is within a factor of 2 of the high affinity K_d for [^{3}H]UK 14,304 binding determined under identical conditions (2.7 ± 0.1 nM). In contrast, in the usual steady state measurements (15 min) or in pre-steady state measurements without agonist preincubation, the EC_{50} values (74 ± 1 and 207 ± 8 nM, respectively) are near the low affinity K_d for [^{3}H]UK 14,304 binding. Reduction of the GTP concentration in steady state adenylate cyclase assays also decreases the EC_{50} for UK 14,304 from 40 ± 5 nM at 10 μM GTP to 14 ± 5 nM with no added GTP. Both sets of experimental observations are accommodated by a complete kinetic model of inhibition in which the high affinity ternary complex of drug, receptor, and G protein leads to the response. Explicit rate parameters are included for agonist binding, receptor-G protein interactions, GTP binding, and hydrolysis. Despite the functional role of the high affinity state of the α_2-receptor in this model, the steady state EC_{50} for agonist-mediated inhibition correlates best with the K_d of low affinity agonist binding in the presence of high levels of GTP. Under conditions in which formation of the high affinity ternary complex is favored, the EC_{50} for responses approaches the high affinity K_d.

Agonist occupation of α_2-adrenergic receptors in a number of cell types including human platelets leads to inhibition of adenylate cyclase (1). This response involves at least three distinct plasma membrane proteins; the α_2-receptor itself, an inhibitory G protein (G_i), and the catalytic subunit of adenylate cyclase (2). Although the exact molecular mechanism by which hormones inhibit adenylate cyclase is unclear, considerable mechanistic information has been obtained from characterization of both plasma membrane (3–6) and reconstituted systems (7–10). These investigations indicate that strong parallels exist between the mechanisms of stimulation and inhibition of adenylate cyclase (2).

α_2-Antagonist binding to platelet plasma membranes is characterized by a single binding affinity (11–14). In contrast, equilibrium α_2-agonist binding to platelet membranes is more complex, suggesting interactions with a heterogenous population of binding sites possessing different affinities for the agonist (11–14). The high affinity agonist binding is selectively reduced by guanine nucleotides and typically consists of 60% of the total α_2-receptors present in platelet membranes (14). The ternary complex model proposed by DeLean *et al.* (15) to explain the allosteric effect of guanine nucleotides on agonist binding to β-adrenergic receptors has also been applied to equilibrium α_2-agonist binding (14, 16, 17). Biochemical studies have provided evidence that the high affinity conformation of the α_2-receptor corresponds to a complex between the α_2-receptor and G_i (18–20). Detailed studies of the kinetics of α_2-agonist binding to platelet membranes are consistent with a modified ternary complex model in which a subpopulation of α_2-receptors does not interact with G_i (17). Thus, agonist binding heterogeneity appears to involve both different conformations of a single receptor and distinct receptor populations (16, 21).

The functional relevance of the different conformations of the α_2-receptor has not been clearly established. The ternary complex model as originally applied to β-adrenergic receptors proposed that the high affinity receptor conformation is functional. Although indirect evidence has supported the applicability of this model to α_2-receptor-mediated inhibition of adenylate cyclase (16), direct demonstration that the high affinity

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ABBREVIATIONS: G proteins, guanine nucleotide binding regulatory proteins; G_i, G protein that mediates hormonal inhibition of adenylate cyclase; G_s, G protein that mediates hormonal stimulation of adenylate cyclase; G_o, G protein isolated from bovine brain with unknown function; DTT, dithiothreitol; UK 14,304, 5-bromo-6-*N*-(2-4,5-dihydroimidazolyl) quinoxaline; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

ternary complex mediates agonist inhibition has been complicated because the guanine nucleotides required for agonist-mediated inhibition also reduce the high affinity agonist binding. Two experimental observations have been difficult to reconcile with the concept that the high affinity α_2-receptor conformation mediates inhibition. First, the EC_{50} for α_2-agonist-mediated inhibition of platelet adenylate cyclase is close to the K_d of low affinity binding (14, 22, 23). Second, measurements of α_2-agonist binding to intact cells reveals a single low affinity population, apparently due to high levels of endogenous guanine nucleotides (23). Although the ternary complex model accounts reasonably well for α_2-agonist binding, it has not adequately addressed the discrepancy between agonist binding and agonist-mediated response.

In this report we describe experimental and theoretical results that quantitatively account for the low potency of α_2-agonists despite evidence for a primary role for the high affinity ternary complex in responses. Pre-steady state studies of adenylate cyclase inhibition in membranes preincubated with agonist before addition of GTP show a good correlation between the EC_{50} for responses and the K_d for the high affinity agonist binding. Also, at low GTP concentrations, the agonist becomes more potent. A kinetic model is described that incorporates the rates of agonist binding and the time-dependent effects of GTP. The model indicates that the usual agreement between the EC_{50} for responses and the low affinity K_d arises from the transient existence of the receptor-G protein complex in the presence of high concentrations of GTP. Under pre-steady state conditions with agonist preincubation or in the presence of low GTP concentrations, the EC_{50} shifts closer to the K_d of the high affinity α_2-agonist binding because the functional high affinity complex accumulates to higher levels.

Materials and Methods

[^{3}H]UK 14,304 (84–88 Ci/mmol) was purchased from New England Nuclear (Boston, MA). [^{3}H]3′,5′-Cyclic AMP and [α-^{32}P]ATP were obtained from Amersham Corp. (Arlington Heights, IL). Oxymetazoline was a gift from Schering Corp. (Kenilworth, NJ) and UK 14,304 was a gift from Pfizer Ltd. (Sandwich, Kent). Forskolin was obtained from Calbiochem-Behring (La Jolla, CA). All other chemicals were reagent grade or better from standard suppliers. Purified human platelet plasma membranes were prepared as described (24) except that the final resuspension of membranes was in 50 mM Tris·HCl, 10 mM $MgCl_2$, 1 mM EGTA, and 1 mM DTT. Membrane aliquots were stored at −70° until use.

Adenylate cyclase assay. Assays were performed as previously described (14) with the addition of 0.5 mM DTT to all solutions. To exclude NaCl from the assay, Tris salts of ATP, GTP, EGTA, and creatine phosphate were used. All incubations contained 10 μM forskolin and 0.02–0.04 mg of membrane protein per tube (0.1 ml). [α-^{32}P]cAMP formed was measured by the method of Salomon *et al.* (25). Blanks, determined by mixing 50 μl of membrane with 100 μl of stop solution before adding [α-^{32}P]ATP and other reaction ingredients, gave values between 60 and 80 cpm. For agonist preincubation experiments, platelet membranes were incubated with agonist for 3–5 min at 30° before initiation of assays with incubation cocktail. For pre-steady state assays without agonist preincubation and for steady state assays, membranes were warmed for 3–5 min at 30° before addition of agonist and incubation cocktail.

[^{3}H]UK 14,304 equilibrium binding. Binding was measured as described (14) except the incubation conditions were modified to be similar to those used for agonist preincubation. Buffer A was used for these incubations; it contained (final concentrations) 50 mM Tris·HCl, 5 mM $MgCl_2$, 3 mM EGTA, and 1 mM DTT, pH 7.6. Incubations were performed for 45 min at 30°. Incubations typically contained 0.1 mg/ml membrane protein as determined by the method of Lowry *et al.* (26). Nonspecific binding was defined as [^{3}H]UK 14,304 binding in the presence of 10^{-5} M oxymetazoline.

Analysis of binding data and agonist dose-response curves. B_{max} and K_d values for [^{3}H]UK 14,304 binding were determined by nonlinear least squares analysis of specific binding using GRAPH PAD (ISI). For the agonist concentrations used, all of the results were best fit by a single population of binding sites. The percentage of inhibition of adenylate cyclase obtained at various agonist concentrations was fit, using the nonlinear least squares curve-fitting program GRAPH PAD, to a sigmoidal function $Y = P_{min} + (P_{max} - P_{min}) \cdot [D^n/(D^n + EC_{50}^n)]$ where P_{min} is the minimum per cent inhibition, P_{max} is maximum per cent inhibition, EC_{50} is the concentration of agonist that produces half maximal inhibition, and n is the Hill coefficient or slope factor. In general the slope factors were less than unity (0.6–0.9). P_{min} was not fixed but was a variable parameter in all analyses. All data are expressed as mean ± standard error unless stated otherwise.

Simulation of agonist dose responses. Theoretical agonist concentration-response functions were simulated on an ATT Unix or a micro VAX using the SAAM 29 kinetic modeling program (27). The concentration of *DR*, *DRG*, *DRG-GTP*, and active G protein (*G*-GTP*) were simulated for times of 15 sec and 15 min according to model 1 (Fig. 1). For simulations of agonist preincubation, a 3-min exposure to agonist with a GTP concentration of zero was included before increasing the GTP concentration to 10^{-5} M to generate active G protein.

The concentration of G protein used in the simulations is 100-fold greater than that of the α_2-receptor, because the molar ratio of pertussis toxin substrate to [^{3}H]yohimbine binding sites is 50–100 or greater for these platelet plasma membrane preparations (14). Because of the excess G protein, equilibrium agonist binding curves predicted by our model will all be hyperbolic (14, 28, 29). The low affinity component of equilibrium agonist binding thus appears to represent a distinct subpopulation of the α_2-receptor that is unable to couple to G_i (14, 17, 21). It is not included in our model (see Discussion); consequently, high and low affinity refer to the conformational states of the single receptor population that is able to interact with G_i.

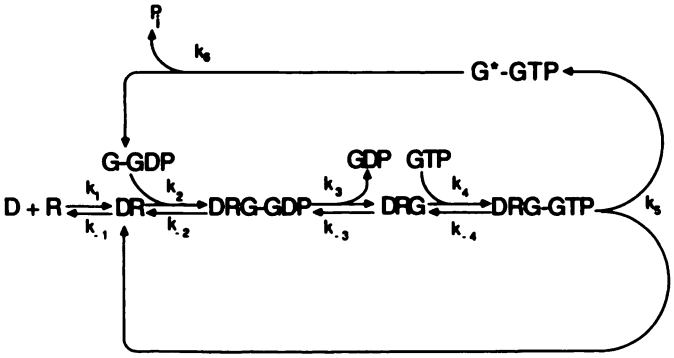

Fig. 1. Model of α_2-agonist-mediated activation of G protein. *D* and *R* represent free agonist and α_2-receptor, which combine to form the low affinity complex, *DR*. Interaction of agonist and receptor facilitates the formation of the complex between agonist, receptor, and GDP liganded G protein, designated as *DRG-GDP*. The high affinity ternary complex *DRG* is formed upon release of *GDP*. *GTP* can then bind to the ternary complex forming *DRG-GTP*, which then dissociates into the low affinity *DR* complex and activated G protein, *G*-GTP*. The inhibition of adenylate cyclase is assumed to be proportional to the concentration of *G*-GTP* formed. The inhibitory cycle is terminated by the hydrolysis of GTP to GDP restoring G_i to an inactive GDP liganded form. The inhibitory cycle continues in the sustained presence of agonist. The following rate constants were used for computer simulations: k_1, 5×10^6 M^{-1}sec^{-1}; k_{-1}, 0.5 sec^{-1}; k_2[G·GDP], 0.1 sec^{-1}; k_{-2}, 0.1 sec^{-1}; k_3, 0.1 sec^{-1}; k_{-3} · [GDP], 1×10^{-4} sec^{-1}; k_4, 1×10^7 M^{-1} sec^{-1}; k_{-4}, 0.10 sec^{-1}; k_5, 0.05 sec^{-1}; and k_6, 0.10 sec^{-1}.

Quantitation of platelet membrane guanine nucleotides. Platelet plasma membranes were extracted with an equal volume of 0.8 N perchloric acid for 15 min at room temperature, followed by centrifugation in an Eppendorf microcentrifuge. The supernatant was then mixed with an equal volume of Freon/trioctyl-*n*-amine, vortexed, and centrifuged. The top aqueous layer was collected and 100-μl samples were separated on an Altex Ultrasil-Ax high performance liquid chromatography ion exchange column using a linear gradient of 0.15–0.6 M NaH_2PO_4, pH 3.9, over a 30-min period. Concentrations of GDP and GTP in the membranes were determined by comparison with authentic standards.

Results

Steady state and pre-steady state UK 14,304 dose-response studies. To test whether preformation of the high affinity ternary complex in the absence of GTP results in an enhanced ability of agonist to inhibit adenylate cyclase, platelet membranes were preincubated for 3–5 min with or without various concentrations of UK 14,304 before the inhibition assay was initiated with GTP, [α-^{32}P]ATP, and other assay ingredients. [^{32}P]cAMP produced was measured at 15 sec or 15 min and the per cent inhibition of adenylate cyclase by various UK 14,304 concentrations was calculated (Fig. 2). The EC_{50} for inhibition of forskolin-stimulated adenylate cyclase during steady state 15-min incubations was 74 ± 1 nM and maximal inhibition was 41 ± 1% (six experiments). With a 15-sec incubation, an EC_{50} value of 207 ± 8 nM was observed, with maximal inhibition of only 31 ± 4% (six experiments). In contrast, preincubation of membranes with UK 14,304 for 3–5 min in the absence of GTP, followed by 15-sec adenylate cyclase assays, gave an EC_{50} value of 6 ± 1 nM (six experiments). The maximal inhibition after preincubation was 35 ± 3%. These results indicate that preincubation with agonist increases its potency for inhibition of adenylate cyclase during pre-steady state measurements.

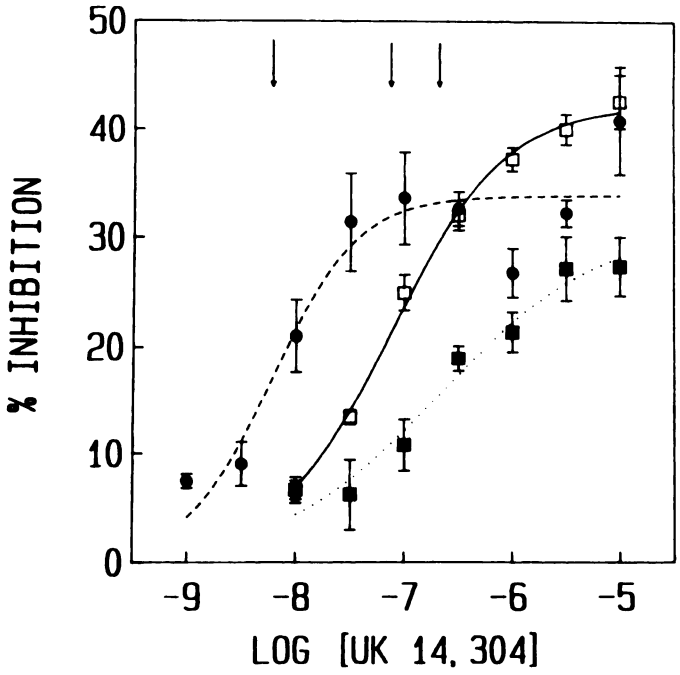

Fig. 2. Steady state and pre-steady state inhibition of platelet adenylate cyclase by UK 14,304. Platelet membranes in buffer A were warmed at 30° for 3 min with (●) or without (■, □) the indicated concentrations of UK 14,304. They were then mixed with an equal volume of adenylate cyclase incubation cocktail containing a concentration of UK 14,304 such that final agonist concentration was as indicated. After incubation at 30° for 15 min (□) or 15 sec (■,●), the reactions were terminated and [^{32}P]cAMP was measured as described in Materials and Methods. For each UK 14,304 concentration, the per cent inhibition of adenylate cyclase was calculated based on the control value without agonist. The control values were 6370 ± 150 pmol/mg (□), 48 ± 5 pmol/mg (■), and 50 ± 7 pmol/mg (●). Each data *point* represents the mean ± standard error of six separate experiments in which quadruplicate data points were determined at each agonist concentration. The data were fit to a sigmoidal function as described in Materials and Methods. *Arrows* indicate EC_{50} values of 6, 74, and 207 nM for pre-steady state assays with agonist preincubation, steady state assays, and pre-steady state assays without preincubation, respectively.

[^{3}H]UK 14,304 high affinity K_d under conditions identical to those used for preincubation. Fig. 3 shows a Scatchard transformation of a [^{3}H]UK 14,304 saturation experiment conducted under conditions identical to those present during agonist preincubation in adenylate cyclase inhibition studies (i.e., 30° and low $MgCl_2$). In the absence of added GTP, the K_d was 2.49 ± 0.47 nM and the B_{max} was 523 ± 82 fmol/mg (three experiments). This B_{max} value was approximately 50% of the number of [^{3}H]yohimbine binding sites previously determined for this platelet plasma membrane preparation. In the presence of 10 μM GTP, a K_d of 3.7 ± 0.9 nM and a B_{max} of 211 ± 5 fmol/mg were obtained. The 70% reduction in the B_{max} of agonist binding by 10 μM GTP is similar to the reduction seen in previous binding studies. Binding both in the presence and absence of GTP was best fit by a single population of high affinity binding sites. This indicates that the agonist binding detected at these concentrations of radioligand corresponds to the high affinity binding sites. The EC_{50} for agonist-mediated inhibition of adenylate cyclase in the preincubation assays at pre-steady state times differed from the high affinity K_d determined in binding studies by only a factor of 2.4.

Models of α_2-agonist-mediated inhibition of adenylate cyclase. A kinetic model of α_2-agonist inhibition, based on the

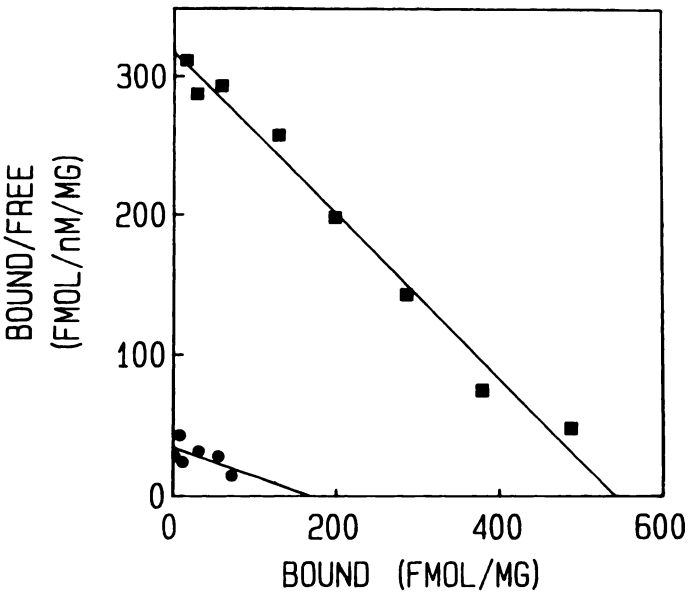

Fig. 3. Scatchard plot of [^{3}H]UK 14,304 binding in the absence and presence of 10 μM GTP. Equilibrium binding of [^{3}H]UK 14,304 (0.5 to 10 nM) to platelet membranes in buffer A was measured in the absence (■) and in the presence (●) of 10 μM GTP as described in Materials and Methods. A Scatchard transformation of specific binding in a single representative experiment is shown. Data are means of triplicate determinations for each agonist concentration.

ternary complex model of β-agonist binding (15) and a related model of β-adrenergic stimulation of adenylate cyclase (30), was evaluated quantitatively (Fig. 1).

We used rate parameter values from several published sources. The rate constants for agonist binding to free receptor (k_1), agonist dissociation from uncomplexed receptor (k_{-1}), and dissociation of the ternary complex (k_6) were obtained from our studies of the kinetics of [^{3}H]UK 14,304 binding to platelet plasma membranes (17). The high affinity agonist binding in our current model is related to formation of the nucleotide free ternary complex *DRG*.[1] Consequently, the rates of formation of that complex (k_3) and the reverse reaction (k_{-3}) were obtained from the related steps, $k_2[N]$ and k_{-2}, in Neubig *et al.* (17). The values of k_3 and k_{-3} are 10- and 3-fold higher, respectively, than those previously published values, to reflect recent data on [^{3}H] UK 14,304 binding at 30°.[2] The rate of GTP binding to the ternary complex (k_4) was obtained from data on GTPγS binding to GDP-depleted G_o (31). Based on the value of k_4 and the EC_{50} for GTP to support α_2-agonist inhibition of adenylate cyclase in the absence of NaCl (10 nM), the rate constant for GTP dissociation (k_{-4}, 0.1 s^{-1}) was obtained from the relation $K_{d_4} = k_{-4}/k_4$. The rate of hydrolysis of GTP (k_6) was taken from studies of GTPase activity in a reconstituted system consisting of partially purified β-adrenergic receptor and purified G_s (32). The least well characterized step in this model is *G-GDP* association with the *DR* complex. The rates $k_2[G\cdot GDP]$ and k_{-2} were selected to give a ratio of 1, resulting in a low agonist affinity for *DRG-GDP*.[1]

Simulations of theoretical UK 14,304 dose-response curves according to this model were done for 15-min (steady state) and 15-sec (pre-steady state) times, with and without a 3-min preincubation of receptor with agonist. The theoretical dose-response relationships for production of *G*GTP* were very similar to those observed experimentally for inhibition of adenylate cyclase (Fig. 4). For simulations of steady state times, the EC_{50} value was 40 nM (experimental value, 74 ± 4 nM). For simulations of pre-steady state assays without agonist preincubation, the EC_{50} value was 104 nM (experimental value, 207 ± 8 nM). Simulation of presteady state *G*-GTP* formation with agonist preincubation yielded an EC_{50} value of 10 nM (experimental value, 6 ± 1 nM). Simulations also generated theoretical maximal levels of *G*-GTP* formation for pre-steady state times with agonist preincubation of 130% of the steady state levels and, at presteady state times without preincubation, 38% of that for steady state conditions. The maximal inhibition observed experimentally for the pre-steady state times with and without preincubation were 85 ± 6% and 51 ± 3%, respectively, of the maximal level of inhibition for steady state measurements. Although the predicted magnitudes of inhibition obtained from simulations are not in exact agreement, the EC_{50} values for *G*-GTP* formation generated from the model agree well with experimental EC_{50} values for inhibition of platelet adenylate cyclase.

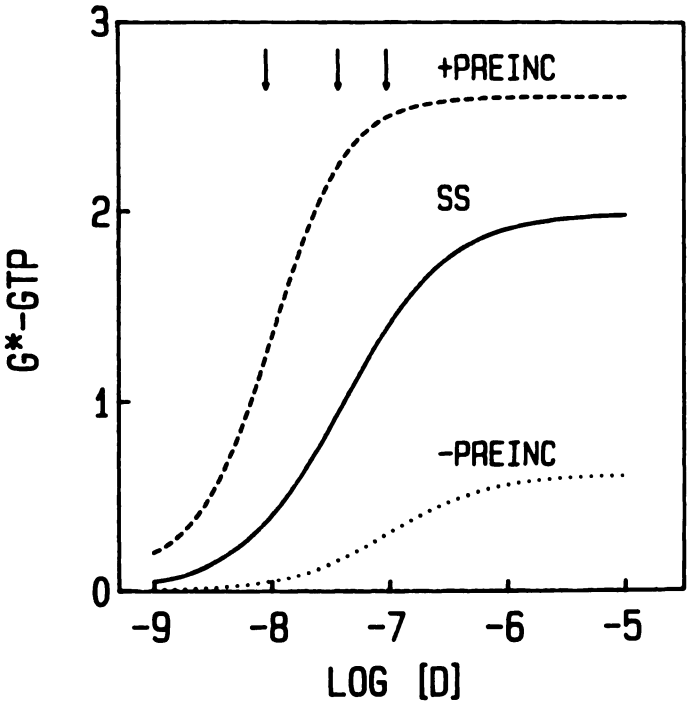

Fig. 4. Simulation of UK 14,304 dose-response curves according to model 1. Simulations of *G*-GTP* formation according to Model 1 were performed using SAAM 29 for steady state (15 min) (——), pre-steady state (15 sec) with agonist preincubation (– – –), and pre-steady state without preincubation (· · · ·). The parameters used are listed in the legend to Fig. 1. G_i and GTP concentrations used in the simulation were 1 nM and 10 μM, respectively. Data were fit to a sigmoidal function and EC_{50} values were obtained as described in Materials and Methods. EC_{50} values of 10, 40, and 104 nM were obtained for pre-steady state with agonist preincubation, steady state, and pre-steady state without agonist preincubation simulations, respectively.

Steady state UK 14,304 inhibition with different GTP concentrations. The ternary complex model suggests that, whereas GTP is required for agonist-mediated inhibition, it will also reduce the stability of the ternary complex and favor the formation of the low affinity agonist-receptor complex. Because of this prediction, we were interested in determining whether theoretical agonist dose-response curves at steady state were influenced by the concentration of GTP present in the adenylate cyclase assay. Simulation of steady state *G*-GTP* formation as a function of UK 14,304 concentration indicates that the maximal amount of *G*-GTP* formed increases in a saturable manner with increasing GTP concentrations (Fig. 5A). However, the EC_{50} for agonist to promote *G*-GTP* formation is also increased with GTP concentration, indicating that the potency of the agonist decreases with increasing GTP concentration. Theoretical agonist EC_{50} values of 5, 25, 38, and 40 nM were obtained in the presence of 1, 10, 100, and 10,000 nM GTP, respectively. An exact solution of differential equations for the model at steady state confirms that, as GTP concentration is increased, the EC_{50} will approach the low affinity K_d of agonist binding (see Appendix) whereas at the limit of zero [GTP] the EC_{50} will equal the high affinity K_d.

We measured inhibition of forskolin-stimulated adenylate cyclase by UK 14,304 with and without the addition of 10 μM

[1] Based on the observation that GDP reduces agonist binding affinity nearly as effectively as GTP, it has been proposed that formation of *DRG·GDP* does *not* result in high affinity agonist binding (2). In dynamic models such as this one it becomes difficult to identify any one "state" of the receptor as having high or low affinity because the overall apparent affinity in equilibrium binding represents a composite or "weighted average" of the individual equilibrium constants (see A15 in Ref. 29 and footnote 3 in Ref. 17). However, for purpose of discussion, we will call *DRG* the high affinity receptor "state" for the following three reasons: 1) there is evidence that direct agonist binding can occur to the *RG* complex (17), 2) biochemical evidence shows that G protein is required to observe high affinity agonist binding (18–20), and 3) inclusion of *DRG* in the binding model results in a high apparent affinity whereas truncation of the model before *DRG* results in a low apparent affinity.

[2] W. J. Thomsen, unpublished observations.

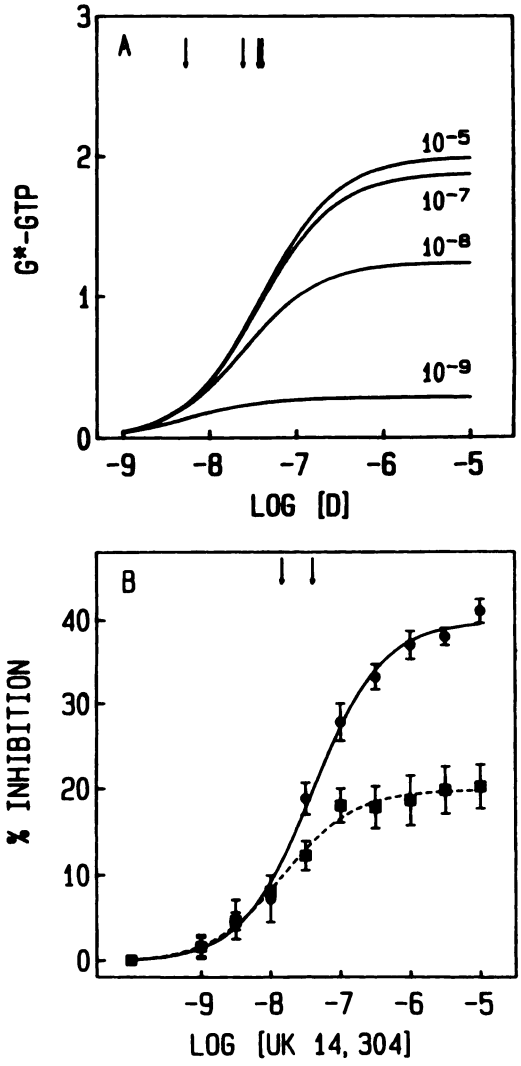

Fig. 5. UK 104,304 dose-response curves for inhibition of platelet adenylate cyclase in the presence of variable GTP concentrations. A, Simulation of UK 14,304 dose-response curves with variable GTP concentrations. Parameters were the same as in Figs. 1 and 4. The concentration of GTP present was 10^{-9} to 10^{-5} M as indicated. Theoretical results were fit to a sigmoidal function and EC_{50} values were estimated as described in Materials and Methods. *Arrows* indicate EC_{50} values of 5, 25, 38, and 40 nM for agonist in the presence of 1, 10, 100, and 10,000 nM GTP. B. Steady state inhibition of platelet adenylate cyclase by UK 14,304 was measured as in Fig. 2 with 10 μM GTP (●) or no added GTP (■). Data *points* represent the mean ± standard error of four experiments performed in triplicate. Data were fit to a sigmoidal function using a nonlinear least squares curve-fitting program as described in Materials and Methods. *Arrows* indicate EC_{50} values of 40 nM for 10 μM GTP and 14 nM for no added GTP.

GTP (Fig. 5B). Without exogenous GTP, the EC_{50} value was 14 ± 5 nM and the maximal per cent inhibition was 20% (four experiments). With the addition of 10 μM GTP, the EC_{50} value and the maximal percentage of inhibition were increased to 40 ± 5 nM and 40%, respectively. Because GTP is an absolute requirement for agonist-mediated inhibition, these results indicate that our platelet plasma membrane preparation contains low but significant levels of *accessible* guanine nucleotide, perhaps in the range of 10–30 nM. Direct high performance liquid chromatography measurements have been performed and 50–100 nM total GTP and ≈2 μM total GDP are carried into the adenylate cyclase assay with the platelet membrane preparation. Addition of the usual high concentration of GTP, 10 μM, to the inhibition assay resulted in a 3-fold reduction in the potency of UK 14,304 whereas the magnitude of inhibition increased 2-fold. These results are in agreement with the predictions of our model.

To understand the origin of these affinity changes it is useful to examine theoretical agonist dose response curves for the steady state levels of *DR*, *DRG*, and *DRG·GTP* in the presence of various concentrations of GTP (Fig. 6). Increasing the GTP concentration resulted in a marked reduction in the maximal steady state levels of *DRG* and an increase in the levels of *DR* and *DRG·GTP*. The ratio of *DRG* to *DR* was reduced from a value of 12 in the presence of 1 nM GTP to a value of 0.001 in the presence of 10 μM GTP. These results indicate that higher levels of *DRG* accumulate in the presence of lower GTP concentrations. The theoretical agonist EC_{50} values for formation of each of the receptor states, *DR*, *DRG*, and *DRG·GTP*, was the same despite the fact that *DR* is considered to be low affinity and *DRG* high affinity. The values were 5, 25, and 40 nM at 1, 10, and 10,000 nM GTP, respectively. These are the same values obtained for formation of *G*·GTP* shown in Fig. 5A. Because of the dynamic interconversions of the different receptor states, at any one GTP concentration, the same concentration of agonist is needed to form half-maximal levels of both the "low affinity" *DR* and the "high affinity" *DRG*. Because the overall EC_{50} can be thought of as a weighted average of the individual K_d values, it is easy to see how conditions that favor accumulation of *DRG* will result in a higher apparent affinity for the formation of all receptor states.

Discussion

In this paper, we have used preincubation studies to provide new evidence that the high affinity conformation of the α_2-adrenergic receptor is linked to production of a biochemical response. The kinetic model of α_2-agonist-mediated inhibition of adenylate cyclase, which incorporates the ternary complex model, accounts for the correlation of the EC_{50} with the K_d of low affinity agonist binding under normal assay conditions, as well as the effect of preincubation on pre-steady state agonist potency in inhibition of adenylate cyclase. The experimental data also agree with the prediction of this model that the potency of agonist to inhibit adenylate cyclase should be greater with lower concentrations of GTP. Thus, for receptors whose mechanisms involve complex dynamic interactions with G proteins, simple correlations between the EC_{50} for response and the high or low affinity K_d cannot identify the functional state of the receptor.

The previously published data supporting the hypothesis that the high affinity conformation of G protein-coupled receptors is associated with the production of responses is indirect. First, a correlation was seen between the efficacy of various agonists to promote stimulation (30) or inhibition (16, 28, 29) of adenylate cyclase in membranes and the ratio of the K_d values for low and high affinity agonist binding. Second, a correlation between agonist efficacy and the ability of guanine nucleotides to reduce high affinity agonist binding has also been observed for adenylate cyclase-coupled receptors (15, 16, 28, 29). However, in intact cell studies, only low affinity agonist binding is usually detected and the K_d for binding is greater than the EC_{50} for agonist regulation of cAMP accumulation (23, 33–35). In membranes, most reports have indicated that the EC_{50} for the

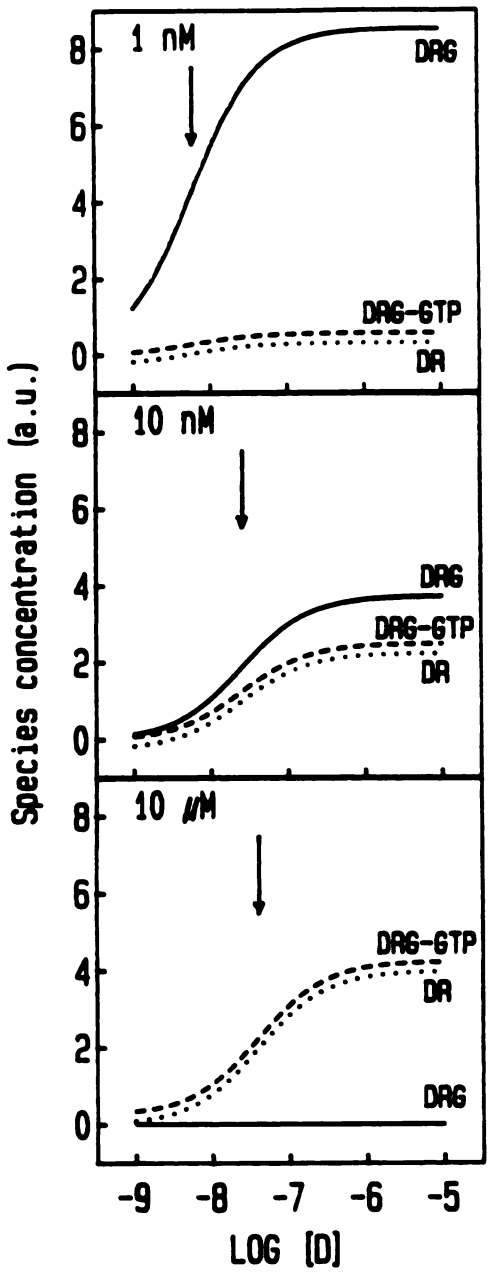

Fig. 6. Simulation of *DR*, *DRG*, and *DRG-GTP* levels as a function of agonist concentration at various concentrations of GTP. Simulations of steady state *DR* (· · · ·), *DRG* (——), and *DRG-GTP* (– – –) concentration in arbitrary units are shown for the indicated agonist concentrations in the presence of 10^{-9} (*top*), 10^{-8} (*middle*), and 10^{-5} M (*bottom*) GTP. Simulations were performed as described in the legend to Fig. 4 and in Materials and Methods. Data were fit to a sigmoidal function and EC_{50} values were obtained as described in Materials and Methods. At any given concentration of GTP, the EC_{50} values for all receptor species, *DR*, *DRG*, and *DRG-GTP*, were the same. EC_{50} values were 5, 25, and 40 nM in the presence of 10^{-9}, 10^{-8}, and 10^{-5} M GTP, respectively. The curves for *DR* (*top* and *middle*) and *DRG·GTP* (*bottom*) are displaced by 0.25 units down and up, respectively, for clarity.

response is similar to the K_d of low affinity agonist binding (13, 14, 29, 36–39). However, in one study of β-adrenergic receptors, reducing the GTP concentration also produced a 3-fold increase in agonist potency, bringing the EC_{50} near the K_d (36). Some exceptions to these more consistent observations have been reported in membrane (40) and intact cell (41) systems. Based on the absence of high affinity agonist binding in many intact cell preparations and the similarity of the EC_{50} to the K_d of low affinity agonist binding for most adenylate cyclase-coupled receptors in membrane systems, it has been suggested that the low affinity conformation is functionally relevant (36, 42). Thus, correlational data can be found to support either the high or the low affinity conformation of the α_2-receptor and other G protein-coupled receptors as the functional form. The interpretation of these data is complicated by differences in experimental conditions between binding and response measurements. Also, a lack of correlation between binding and response can occur when receptors act catalytically to activate effector systems (e.g., spare receptor phenomenon). Thus careful attention to experimental conditions is required and quantitative modelling of these systems may be necessary to account for the observed relations between binding and responses.

Several models of hormonal stimulation of adenylate cyclase have been described to integrate agonist binding and responses. One of the first, the nonstoichiometric floating receptor model of Jacobs and Cuatrecasas (43), accounted for the phenomenon of spare receptors in adenylate cyclase stimulation. Tolkovsky and Levitzki (44) proposed the collision coupling model, in which the β-receptor acts catalytically to activate the adenylate cyclase catalytic subunit. Subsequent models of hormonal stimulation of adenylate cyclase have explicitly included the G protein, which mediates both the effects of guanine nucleotides on agonist binding and agonist stimulation of adenylate cyclase. An extended version of the collision coupling model, which assumes that G protein is permanently coupled to the catalytic subunit of adenylate cyclase, does not account for multiple affinity states of the receptor (45). Stadel and collaborators (30) combined the ternary complex model of agonist binding and the GTPase cycle described by Cassel and Selinger (46) into a unifying model of β-agonist stimulation of adenylate cyclase. However, quantitative evaluation of that model was not reported.

In a model specifically including receptor conformational changes and catalytic activation of G proteins, Swillens and Dumont (47, 48) examined the relation between EC_{50} and K_d. They showed that in all cases the EC_{50} was less than the K_d for low affinity binding and the discrepancy was greatest for drugs with high efficacy. Their model, however, assumed that there was no accumulation of the *DRG* ternary complex and our experimental conditions, (i.e., preincubation with agonist in the absence of GTP and low GTP concentrations in steady state assays) would specifically favor accumulation of the ternary complex (see also Appendix). In addition, their model was not evaluated over a range of GTP concentrations; thus, it does not specifically address the influence of GTP on agonist binding and response.

In this paper, we extend the ternary complex model of agonist binding to include GDP release, GTP binding, and activation of G_i in a kinetic model similar to that of Stadel *et al.* (30). Our model is used to explain the following three experimental observations: 1) that the steady state EC_{50} for agonist-mediated inhibition of adenylate cyclase is similar to the K_d of low affinity agonist binding, 2) that the EC_{50} for inhibition in pre-steady state measurements after preincubation of membranes with agonist is reduced to a value similar to the K_d of high affinity agonist binding, and 3) that the EC_{50} for steady state inhibition is reduced at lower concentrations of GTP. Our model, in which the high affinity α_2-receptor G protein complex (*DRG*) is the

direct precursor of active G_i, accounted for all of these experimental observations. An alternative model, which assumes that the high affinity conformation of the α_2-receptor is desensitized as is seen for the nicotinic cholinergic receptor (49), did not even qualitatively agree with the results (data not shown).

In our current model of agonist-mediated inhibition of adenylate cyclase, we have not included either the "uncoupled" low affinity α_2-receptor (14, 17, 21) or the "precoupled" high affinity α_2-receptor-G_i complex described recently (17). The uncoupled low affinity receptor is unlikely to contribute to inhibition of adenylate cyclase because it does not exhibit affinity changes in the presence of GTP. The data presented here show that both the α_2 response and the population of α_2-receptors capable of high affinity agonist binding undergo similar affinity changes with variations in GTP concentration. We excluded the "precoupled" α_2-receptors to avoid further complicating an already complex model. We would not have expected any influence of precoupled receptors on simulations of either agonist preincubation or steady state results. In the former case, formation of the *DRG* complex would be induced during preincubation with agonist whether or not a precoupled *RG* complex existed. In steady state measurement, the high GTP concentrations would destabilize the precoupled complex. The one situation in which evidence of the precoupled complex might have been expected to be found is the pre-steady state experiments without agonist preincubation. However, the simultaneous addition of 10 μM GTP could promote dissociation of the *RG* complex before agonist could bind. Studies of the time course of the preincubation effect and appearance of high affinity agonist binding are in progress to test the function of the precoupled α_2-receptor-G protein complex.

The values of kinetic parameters used for our simulations were obtained from various sources (see Results). The parameters for GTP binding (k_4, k_{-4}) and GTPase (k_6) reactions were taken from data on solubilized G_o (31) and β-receptor stimulated G_s (32), respectively, and may not represent the actual values for the platelet α_2-adrenergic system. These values, however, represent reasonable approximations, and 10-fold variations in the rate constants for GTP binding to the ternary complex (k_4) and the GTPase step (k_6) did not affect the EC_{50} values for steady state and agonist preincubation simulations.[2] Variations in the rates of agonist-receptor and G protein interaction (k_2), GDP release (k_3), and dissociation of the ternary complex (k_5) did affect the relative EC_{50} values. Ten-fold reductions in k_2 and k_3 eliminate the decrease in EC_{50} with agonist preincubation. At higher values of those parameters, the pre-steady state EC_{50} with agonist preincubation decreased whereas that for steady state did not change, thereby increasing the difference between the two EC_{50} values.[2] At low values of k_5, the EC_{50} values were also similar but, as the value was increased, the steady state EC_{50} increased to enhance the difference between the two EC_{50} values.[2] These results indicate that the rates of receptor-G protein interaction, GDP release, and dissociation of ternary complex are most important in determining whether the EC_{50} for agonist preincubation will be less than that for steady state simulations.

The primary discrepancy between experimental and theoretical results concerns the magnitude of the responses for the steady state and pre-steady state groups. Simulations predicted that the maximal inhibition for agonist preincubation would be higher than that for steady state measurements. The maximal level of inhibition observed for preincubation measurements was slightly lower. This discrepancy could be a result of inaccurate rate parameters used in simulations. Indeed, differences in magnitudes of *G*-GTP* formation were apparent between simulation groups when rates of k_2, k_3, k_5, and k_6 were varied.[2] Actual measurements of the rate parameters used in this model may help to generate theoretical data that are closer to experimental data (50, 51). Also, the assumption that there is a strict linear relation between *G*-GTP* formation and adenylate cyclase inhibition may not be correct.[3] Finally, some α_2-receptors may undergo desensitization during preincubation with agonist, resulting in a diminished response. Despite this discrepancy, the alternate model that postulates that the high affinity conformation is desensitized does not even qualitatively account for experimental data.

This report addresses experimental and theoretical problems inherent in the analysis of ligand binding and responses for G protein-coupled receptors. It provides strong evidence that the high affinity conformation of the α_2-receptor is functionally relevant. Our kinetic model of α_2-agonist inhibition explicitly includes rate parameters from the literature and adequately accounts for our experimental data. This model explains the observation that, although the high affinity conformation of the α_2-receptor is more closely related to activation of G protein, the EC_{50} for agonist response is closer to the K_d of low affinity binding under normal assay conditions.

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Appendix

In order to more completely explore the effects of [GTP], the steady state solutions for the differential equations describing our model of α_2-receptor-mediated G protein activation were solved exactly. The definitions of the rate constants are as in Fig. 1. In the following equations, $[D]$ is the free concentration of drug, $[G_T]$ is the total concentration of G protein, $[R_T]$ is the total receptor concentration, $[R]$ is the concentration of uncomplexed receptor and $[DR]$, $[DRG{\cdot}GDP]$, $[DRG]$ and $[DRG{\cdot}GTP]$ are the concentrations of the various molecular complexes formed. $[G^*{\cdot}GTP]$ is the concentration of the activated G protein species. It is assumed that $[G{\cdot}GDP]$ is always in excess over $[R_T]$ and $[G^*{\cdot}GTP]$; consequently $[G{\cdot}GDP] \simeq [G_T]$. We do not attempt in this model to identify the molecular nature of the active state but it could represent the dissociated $\alpha{\cdot}$GTP and $\beta\gamma$ subunits of the G protein.

The differential equations describing the model can be ex-

[3] Preliminary experiments characterizing the relation of α_2-receptor occupancy and response using the specific irreversible alkylating agent benextramine have indicated that substantial receptor reserve is not present for platelet membrane α_2-receptors. Treatment of membranes with 50–80 μM benextramine resulted in a 60 ± 17% reduction in the B_{max} for [^{3}H]yohimbine binding and a 39 ± 17% decrease in maximal inhibition with 10 μM epinephrine. These observations suggest that substantial receptor reserve or excess *G*-GTP* do not occur.

pressed as follows:

$$d[R]/dt = -k_1[D]\,[R] + k_{-1}\,[DR] \quad \text{(A1)}$$

$$d[DR]/dt = k_1[D]\,[R] + k_{-2}[DRG\cdot GDP] + k_5[DRG\cdot GTP]\,(k_{-1} + [G_T]k_2)\,[DR] \quad \text{(A2)}$$

$$d[DRG\cdot GDP]/dt = k_2[DR][G_T] + k_{-3}[DRG]\,[GDP] - (k_{-2} + k_3)\,[DRG\cdot GDP] \quad \text{(A3)}$$

$$d[DRG]/dt = k_3[DRG\cdot GDP] + k_{-4}[DRG\cdot GTP] - (k_{-3}[GDP] + k_4[GTP])[DRG] \quad \text{(A4)}$$

$$d[DRG\cdot GTP]/dt = k_4[DRG][GTP] - (k_{-4} + k_5)[DRG\cdot GTP] \quad \text{(A5)}$$

$$d[G^*\cdot GTP]/dt = k_5[DRG\cdot GTP] - k_6[G^*\cdot GTP] \quad \text{(A6)}$$

At steady state the derivatives are all zero and the solutions for equations A1–A6 are:

$$[R] = [R_T] - [DRG\cdot GDP] - [DR] - [DRG] - [DRG\cdot GTP] \quad \text{(A7)}$$

$$[DR] = \{k_{-2}[GDP]k_{-3}(k_{-4} + k_5) + (k_{-2} + k_3)[GTP]k_4k_5\} \cdot \frac{[D]k_1}{\Delta} \cdot [R_T] \quad \text{(A8)}$$

$$[DRG\cdot GDP] = [G_T]k_2\{[GDP]k_{-3}(k_{-4} + k_5) + [GTP]k_4k_5\} \cdot \frac{[D]k_1}{\Delta} \cdot [R_T] \quad \text{(A9)}$$

$$[DRG] = [G_T]k_2k_3(k_{-4} + k_5) \cdot \frac{[D]k_1}{\Delta} \cdot [R_T] \quad \text{(A10)}$$

$$[DRG\cdot GTP] = [G_T]k_2k_3[GTP]k_4 \cdot \frac{[D]k_1}{\Delta} \cdot [R_T] \quad \text{(A11)}$$

$$[G^*\cdot GTP] = \frac{k_5}{k_6} \cdot [G_T]k_2k_3[GTP]k_4 \cdot \frac{[D]k_1}{\Delta} \cdot [R_T] \quad \text{(A12)}$$

where

$$\Delta = [D]k_1[\{(k_{-2} + [G_T]k_2)[GDP]k_{-3} + [G_T]k_2k_3\}(k_{-4} + k_5) + \{[G_T]k_2k_3 + (k_{-2} + [G_T]k_2 + k_3)k_5\}[GTP]k_4] + k_{-1}[k_{-2}[GDP]k_{-3}(k_{-4} + k_5) + (k_{-2} + k_3)k_5[GTP]k_4] \quad \text{(A13)}$$

Thus $[G^*\cdot GTP]$ at steady state can be expressed in the following form.

$$[G^*\cdot GTP] = \frac{[D]\cdot[G^*\cdot GTP]_{max}}{K_{app} + [D]} \quad \text{(A14)}$$

From A12–A14, the maximum response ($[G^*\cdot GTP]_{max}$) and the concentration of agonist needed to elicit a half maximal response (K_{app}) can be easily calculated.

$$[G^*\cdot GTP]_{max} = \frac{(k_5/k_6)[G_T]k_2k_3[GTP]k_4[R_T]}{[\{(k_{-2} + [G_T]k_2)\,[GDP]k_{-3} + [G_T]k_2k_3\}(k_{-4} + k_5) + \{[G_T]k_2k_3 + (k_{-2} + [G_T]k_2 + k_3)k_5\}[GTP]k_4]} \quad \text{(A15)}$$

$$K_{app} = \frac{k_{-1}}{k_1} \frac{k_{-2}[GDP]k_{-3}(k_{-4} + k_5) + (k_{-2} + k_3)k_5[GTP]k_4}{[\{(k_{-2} + [G_T]k_2)[GDP]k_{-3} + [G_T]k_2k_3\}(k_{-4} + k_5) + \{[G_T]k_2k_3 + (k_{-2} + [G_T]k_2 + k_3)\,k_5\}[GTP]k_4]} \quad \text{(A16)}$$

At the limit of $[GTP] \rightarrow 0$

$$K_{app} = \frac{k_{-1}}{k_1} \frac{k_{-2}}{k_{-2} + [G_T]k_2(1 + (k_3/[GDP]k_{-3}))} \quad \text{(A17)}$$

which is equal to the high affinity K_d for drug binding in the absence of GTP.

It should be noted that inclusion of the $DRG\cdot GDP$ state in the model makes the K_{app} as well as the predicted high affinity K_d dependent on $[GDP]$ at low concentrations of GTP.

At the limit of $[GTP] \rightarrow \infty$

$$K_{app} = \frac{k_{-1}}{k_1} \frac{k_5}{k_5 + [G_T]k_2\{(k_3 + k_{-2})\}} \quad \text{(A18)}$$

which is equal to the low affinity K_d for drug binding in the presence of high concentrations of GTP. It should be noted that the second term in A18 is always less than 1. So, in this kinetic model, the K_d for agonist binding in the presence of saturating GTP concentrations must be less than that expected for binding to the uncomplexed receptor alone (i.e., k_{-1}/k_1). The parameter values used in these simulations give a K_{app} of 0.4 times that expected for binding to uncomplexed receptor alone. These results show that the high GTP concentrations necessary for maximal agonist responses reduce the potency of the agonists such that EC_{50} values are close to those of the low affinity K_d. When suboptimal GTP concentrations are used, the EC_{50} for agonist approaches the high affinity K_d.

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