Allosteric equilibrium model explains steady-state coupling of β -adrenergic receptors to adenylate cyclase in turkey erythrocyte membranes

Özlem UGUR and H. Ongun ONARAN*

Department of Pharmacology, Medical Faculty of Ankara University, Sıhhiye 06100, Ankara, Turkey

We used a simple experimental approach to clarify some contradictory predictions of the collision coupling and equilibrium models (e.g. ternary complex, two-state ternary complex or quaternary complex), which describe G-protein-mediated β -adrenergic receptor signalling in essentially different manners. Analysis of the steady-state coupling of β -adrenoceptors to adenylate cyclase in turkey erythrocyte membranes showed that: (1) in the absence of an agonist, Gpp(NH)p (a hydrolysis-resistant analogue of GTP) can activate adenylate cyclase very slowly; (2) this activity reaches a steady state in approx. 5 h, the extent of activity depending on the concentration of the nucleotide; (3) isoprenaline-activated steady-state adenylate cyclase can be inactivated by propranolol (a competitive antagonist that relaxes the receptor activation), in the presence of Gpp(NH)p (which provides a virtual absence of GTPase) and millimolar concentrations of Mg^{2+} (the rate of this inactivation is relatively fast); (4) increasing the concentration of Gpp(NH)p can saturate the steady-state activity of adenylate cyclase. The

saturated enzyme activity was lower than that induced by isoprenaline under the same conditions. This additional agonist-induced activation was reversible. In the light of these results, we conclude that agonist can also activate the guanine nucleotide-saturated system in the absence of GTPase by a mechanism other than guanine nucleotide exchange. We explain these phenomena in the framework of a quaternary complex model as an agonist-induced and receptor-mediated dissociation of guanine nucleotide-saturated residual heterotrimer, the equilibrium concentration of which is not necessarily zero. These results, which suggest a continuous interaction between receptor and G-protein, can hardly be accommodated by the collision coupling model that was originally suggested for the present experimental system and then applied to many other G-protein systems. Therefore we attempt to unify the equilibrium and collision coupling approaches to provide a consistent theoretical basis for the G-protein-mediated β -adrenergic receptor signalling in turkey erythrocyte membranes.

INTRODUCTION

Heptahelical transmembrane receptors and their immediate partners, the guanine nucleotide-binding regulatory proteins (G-proteins), constitute an important transmembrane signalling machinery in almost every living cell. The exchange of GDP and GTP on the α subunit of G-protein with the 'aid' of agonist-bound receptor, the dissociation of the GTP-bound form of G-protein into its α and $\beta\gamma$ subunits, which carry the signal to the cellular effectors, and the intrinsic GTPase of $G\alpha$, which restores the inactive state, define the life cycle of this system descriptively [1–3]. From a theoretical point of view, however, there are two approaches that typically dominate both the currently accepted theoretical models and the intuitively constructed paradigm for the activation of this signalling system. These are the ternary complex idea (and its extensions) and the catalytic collision coupling mechanism. The ternary complex model, which describes the interaction between ligand, receptor and G-protein in thermodynamic equilibrium, was first formulated and used to explain the binding properties of β -adrenergic ligands to β -adrenoceptors (β -ARs) [4] and then extended to a quaternary [5] and a quaternary [6] complex model to accommodate the current phenomenology of G-protein-mediated transmembrane signalling. Recently, to explain the behaviour of activating mutations in β -adrenoceptors, Samama et al. [7] further extended this idea by including two different receptor states (i.e. active and inactive) into the ternary complex model. In all these models, the protein components are considered to be allosterically regulated molecules. These allosteric regulations are described by using the

reciprocity principle [8]. In the simplest form of these models, ligand and G-protein are assumed to bind to two distinct sites on the receptor protein to form a ternary complex, and to modify the binding affinity of each other reciprocally when they are simultaneously bound to the receptor [9,10]. The collision coupling model, in contrast, sees the receptor protein as a freely diffusing catalyst that has access to a number of G-protein molecules in the plane of the cell membrane, so that it can activate more than one G-protein molecule in unit time [11,12]. This catalytic view of the receptor action has been proposed particularly to explain the steady-state stoichiometric signal amplification at the G-protein level, and has been modified and revised until recently to provide a more refined model [13–16]. This model describes the activation as depending on (1) the probability of collision (or encounter) of an 'active' receptor with an inactive G-protein, and (2) the probability of this collision's being fruitful. Inactivation, in turn, is determined independently from the activation process by a constant rate of GTPase, to confer a steady-state signal amplification in the above sense. Thus the analysis covers the stationary non-equilibrium or non-stationary states of the system.

Although the ideas behind the ternary complex and collision coupling models have been widely used (explicitly or implicitly) in the interpretation of experimental data and accepted equally well by 'receptorologists', they have been developed quite independently to explain different aspects of receptor action. Consequently, consistency of the postulates of the ternary complex and the collision coupling models and the way in which they describe the signal transduction system, have not been stated

Abbreviations used: β -AR, β -adrenergic receptor; G-protein, GTP-binding regulatory proteins; Gpp(NH)p, 5'-guanylylimidodiphosphate; GTP[S], guanosine 5'-[γ -thio]triphosphate

* To whom correspondence should be addressed.

explicitly on a comparative basis. Apart from the fact that different physical states of the signalling system are dealt with by the collision coupling and ternary complex models (non-equilibrium and equilibrium respectively), they predict different results for the same experimental situation. In an experiment where non-hydrolysable analogues of GTP are used instead of GTP, the collision coupling model predicts that the activation of G-protein (consequently the associated effector) will be maximum independently of the extent of receptor activation (i.e. the occupancy number by an agonist) when the system is in equilibrium. This prediction is due to the crucial assumption of the collision coupling model that activation and inactivation processes are independent and irreversible events [15,16]. This assumption is needed to accommodate a steady-state stoichiometric signal amplification of the G-protein level. Thus, in the absence of GTPase, the collision coupling idea with this assumption implies that the receptor will be unable to receive and transduce the strength of the signal when the system is in thermodynamic equilibrium. The equilibrium models, in contrast, deal with the problem of how the signal strength is actually transduced in thermodynamic equilibrium. This single disagreement can be viewed as a source of contradiction for the simultaneous validity of these two approaches.

Although there exists direct or indirect experimental evidence for [11,17,18] or against [19–23] the steady-state predictions of the collision coupling model, they have never been analysed explicitly in a unifying manner. In this study we addressed these questions by testing experimentally the steady-state predictions of the present form of the collision coupling model. Results showed that such testable predictions of the collision coupling mechanism that necessarily invalidate the equilibrium models do not meet the experimental data in turkey erythrocyte membranes. We used the β -AR– G_s –adenylate cyclase system of turkey erythrocyte membrane as an experimental tool for two reasons: (1) historically, both the collision coupling and the ternary complex models have their origin in the experimental data that have been obtained in this experimental system, and (2) it is a simple and prototypical tool that has been widely used to investigate G-protein-mediated β -AR signalling [24,25].

MATERIALS AND METHODS

Models and computer simulations

We used the quinternary complex model for equilibrium calculations. Details of the model have been given elsewhere [6]. Briefly, the model describes the equilibrium interactions of three protein species (receptor, $G\alpha$ and $G\beta\gamma$) with each other and with their ligands; namely, guanine nucleotides that bind to a nucleotide-binding site on $G\alpha$, and hormonal ligands that bind to a ligand-binding site on the receptor molecule. The $G\beta\gamma$ subunit binds to another distinct site on $G\alpha$. No direct binding between receptor and $G\beta\gamma$ was assumed in the present calculations. The following equilibria describe the bimolecular interactions:

$$[HR] = K[H][R]$$

$$[G\alpha N] = J[G\alpha][N]$$

$$[RG\alpha] = M[R][G\alpha]$$

$$[G\alpha G\beta\gamma] = L[G\alpha][G\beta\gamma]$$

where H, R and N signify hormones, receptor and guanine nucleotides respectively. J , K , L and M stand for unconditional affinities for the corresponding equilibria. Conditional affinities that govern the formation of multivalent species in equilibrium (i.e. $HRG\alpha$, $RG\alpha G\beta\gamma$, $G\alpha NG\beta\gamma$, $RG\alpha N$, $RG\alpha NG\beta\gamma$,

$HRG\alpha G\beta\gamma$, $HRG\alpha N$, $HRG\alpha NG\beta\gamma$) are constructed by using the coupling factors as described previously. These coupling factors are thermodynamic measures of the effects that two (or more) ligands exert on the binding of each other when they are simultaneously bound to the same species. Unconditional affinities and the coupling factors that govern the above-mentioned equilibria are shown schematically in Figure 1. Although the quinternary complex model envisions higher-order interactions between the binding sites on the protein species, we show in Figure 1 only those interactions that have been manipulated for the present purposes. We numerically solved the equations of equilibria (which are bounded with the conservation of mass), for the equilibrium concentrations of all possible free and complexed species. For simulating the experimental data we defined activation and ligand binding in the following manner: activation is the total concentration of all species that contain $G\beta\gamma$ -free and GTP-bound $G\alpha$, and ligand binding is the total concentration of all ligand-bound species.

For the collision (or encounter) coupling model we used the expressions for the activation (k_a) and inactivation (k_i) rates given by Stickle and Barber [15,16]. Activation rate is defined as the product of P and ω , where P is the average probability of encounters of average duration τ between receptor and G-protein resulting in activation and ω is the frequency of encounters. P is a function of the rate constants for association and dissociation of the agonist–receptor complex (k_{on} and k_{off}), the survival rate of an encounter ($k_s = \tau^{-1}$) and the rate constant for the activation of G-protein in an encounter with an active receptor (k_e). The last of these is given as the efficiency of an encounter, which we manipulated to simulate different concentrations of guanine nucleotide. The parameters of the encounter–collision coupling model are shown schematically in Figure 2.

We always set the inactivation rate to zero, assuming that there is no GTPase in the present experimental conditions. We set the activation parameters arbitrarily, regarding the general nature of the predictions that we wish to make as being independent of the parameter setting.

Preparation of cell membranes

We adopted and slightly modified the procedure described by Stadel et al. [26] for the preparation of turkey erythrocyte membranes. Briefly, erythrocytes were obtained from adult turkeys in PBS. Lysis and homogenization of the washed erythrocytes were performed in 10 mM Tris/5 mM $MgCl_2$ /0.2 mM PMSF/1 mM EGTA/2 mM dithiothreitol at pH 7.4. The lysate was centrifuged at 39000 g (10 min) and the pellet was resuspended in 30 mM Tris/10 mM $MgCl_2$ /0.2 mM PMSF/1 mM EGTA at pH 7.4. After discarding the 400 g (10 min) pellet, the supernatant was layered on a discontinuous sucrose gradient (25% and 40%, w/w) prepared in the same buffer and centrifuged at 1200 g for 10 min. The band at the 25/40% interface was removed and stored in the same buffer containing 25% sucrose at an approximate protein concentration of 2 mg/ml, at $-70^\circ C$ until used.

Adenylate cyclase assay and determination of cAMP

Diluted membranes were incubated at $37^\circ C$ in Tris buffer (in mM, 50 Tris, 100 KCl, 2 $MgCl_2$) with the indicated concentrations of the guanine nucleotides and drugs. Adenylate cyclase assay was started at given time points by adding to the incubation tubes an adenylate cyclase buffer containing, in addition to its own constituents, identical chemicals at identical concentrations to the incubation buffer. The total volume of the cyclase assay was 100 μl with 7–10 μg of membrane protein. The common

Figure 1 Schematic representation of equilibrium quaternary complex model

H, R, N, G_α and $G_{\beta\gamma}$ signify hormone, receptor, nucleotide (GDP or GTP) and α and $\beta\gamma$ subunits of G-protein respectively. Unconditional affinity constants for the formation of HR, RG_α , $G_\alpha G_{\beta\gamma}$ and NG_α complexes are designated K, M, L and J respectively. α , β , δ , ϕ stand for possible heterotropic interactions that may take place between the indicated binding sites. Higher-order interactions are not shown.

constituents of the cyclase buffers were (in mM final concentrations) 50 Tris, 100 KCl, 1.6 $MgCl_2$, 0.5 ATP, 1 isobutylmethylxanthine, 5 phosphoenolpyruvate and 0.01 mg/ml pyruvate kinase (5 units/ml). The assay was stopped by adding 100 μ l of 0.2 M HCl after 10 min. Samples were stored at 4 °C until assayed for cAMP. Accumulation of cAMP per mg of membrane protein in unit time is given as the adenylate cyclase activity for the indicated time and conditions. We also considered this activity as a reflection of the extent of G_s activity, assuming that at the saturating concentration of ATP we were measuring the V_{max} of the adenylate cyclase that is regulated by G_s [27,28]. In preliminary experiments we showed that the accumulation of cAMP, even at the highest enzyme activity induced by NaF + $AlCl_3$, was apparently linear for 10 min. Because we did not deal with the exact pattern of the kinetics of the adenylate cyclase activation, we did not make any correction for the initial rate of enzyme activity. One might think that the change in the enzyme activity might be so fast that it could lead to a discrepancy between the initial and the average enzyme activities during 10 min of assay. Nevertheless, the observed rate of adenylate cyclase activation was relatively slow in most cases, compared with the duration of the assay.

We tested the stability of adenylate cyclase or G_s during the long incubation periods by measuring the adenylate cyclase activity before and after incubating the membranes under stimulus-free conditions. We observed no significant loss of reactivity during this period.

cAMP was determined by radioimmunoassay with the acetylation protocol [29]. High-affinity rabbit anti-cAMP antibodies were raised in our laboratory by using BSA-conjugated cAMP. Succinyl-cAMP tyrosine methylester (ScAMP-TME) was iodinated by the chloramine-T method. Mono- and di-iodo ScAMP-TME which were used as tracer ligands for radioimmunoassay, were purified by gel-filtration chromatography (Sephadex G25 superfine, equilibrated and eluted with 1 M sodium acetate, pH 5) [30].

Figure 2 Schematic representation of (encounter) collision coupling model

R and G signify receptor and G-protein respectively. The asterisk indicates the active forms of the corresponding species. According to this model, receptor diffuses freely relative to G-protein (indicated as an irregular path), and it toggles randomly between active and inactive forms along this path, which is governed by the rate constants k_{on} and k_{off} [k_{on} may have a unit of $time^{-1}$ or $(mol \cdot time)^{-1}$ depending on whether the activation is assumed to be spontaneous or agonist-induced]. During its diffusion, receptor enters the encounter zone (indicated as a dotted circle around the G-protein) with a certain average frequency (ω). Each encounter may last τ_i seconds, which yields an average encounter time of τ . During an encounter, an inactive G-protein might become active (i.e. nucleotide exchange might occur) with a rate constant of k_e , provided that the encounter takes place between an active receptor and an inactive G-protein. The overall rate of receptor-induced G-protein activation (k_a) is determined by these parameters (k_{on} , k_{off} , ω , τ , k_e). Inactivation, in contrast, is determined by an independent rate of k_i .

Binding experiments and other procedures

We assessed the ligand binding to β -AR by the standard filtration method. Diluted membranes (7 μ g per tube) were incubated in a final volume of 100 μ l in a Tris buffer [50 mM Tris/100 mM KCl or NaCl/0.1 % BSA (pH 7.4)] with the indicated concentrations of a competitor ligand and [^{125}I]cyanopindolol (20000 c.p.m. per tube) at room temperature for 150 min. The reaction was stopped by adding 3 ml of ice-cold buffer and filtering through a Whatman GF/B filter under vacuum, using a 12-well filtration manifold (Millipore 1225). The filters were then washed quickly with 15 ml of cold buffer. Radioactivity on the filters was considered as bound ligand.

A four-parameter logistic equation:

$$y = \frac{(a-d)}{1 + (x/c)^p} + d$$

was used to fit the nucleotide concentration (x) against steady-state adenylate cyclase activity (y) curves, or competition binding curves. A simple exponential growth equation, $y = a(1 - e^{-kt})$, or an empirical logistic equation, $y = a - a\{[1 + e^{-b(t-c)}]^d\}^{-1} + m$, was used to fit the time-dependent data of activation. Goodness of fit, wherever applicable, was assessed by using F or χ^2 statistics

based on the likelihood theory. The accuracy of nonlinear estimates was given in terms of standard errors that were calculated from the diagonals of asymptotic variance–covariance matrices.

Purity and stability of the nucleotides during the long incubation periods were assayed by HPLC (Jasco) with a weakly basic anion-exchanger column (Waters, Protein-Pak DEAE-5PW). Separation of nucleotides was achieved by a continuous NaCl and pH gradient in 5 mM phosphate buffer. We found less than 0.5% initial GTP as a contaminant in the 5'-guanylylimidodiphosphate [Gpp(NH)p] preparations. Gpp(NH)p was stable over the present experimental conditions, unlike guanosine 5'-[γ -thio]triphosphate (GTP[S]). Therefore we avoided the use of GTP[S] in the present experiments. Protein concentration was determined by the methods of Lowry [31] and Bradford [32], with BSA as standard.

Chemicals

All nucleotides and dithiothreitol were from Boehringer Mannheim. Propranolol was a gift from Dogu Pharmaceuticals (Istanbul). [125 I]Cyanopindolol (2000 Ci/mmol) and Na 125 I (2000 Ci/mmol) were obtained from Amersham. All other chemicals were purchased from Sigma at highest purity. Concentrated stock solutions of isobutylmethylxanthine and PMSF were prepared in 90% (v/v) ethanol and that of forskolin in DMSO.

RESULTS

Time- and concentration-dependent activation of adenylate cyclase in the presence of Gpp(NH)p and in the absence of agonist

The original formulation of the collision (or encounter) coupling model denies the activation of G-protein (hence the effector) in the absence of an agonist. However, it can be modified by allowing the receptor to toggle spontaneously between an active and an inactive conformation, as suggested by Stickle and Barber [16] (see Figure 2). This manipulation does not change the general nature of the idea but does admit a background activation in the presence of guanine nucleotides as though the receptor were activated by an agonist. In the absence of an inactivation process, this idea predicts that the activation will be maximal at equilibrium, irrespective of the concentration of guanine nucleotide in the medium. This statement is equivalent to the well-known prediction of the collision coupling model that in the absence of an inactivation process, the steady-state activity of G-protein is maximal and is independent of the number of receptors occupied by agonist. In other words, the equilibrium activity of the G-protein is independent of the agonist concentration. We tested this hypothesis experimentally by using Gpp(NH)p as the activator guanine nucleotide. Membranes were incubated at different concentrations of Gpp(NH)p in the absence (Figure 3A) or presence (Figure 3B) of 1 μ M GDP, and adenylate cyclase activity was determined at the indicated time points. A very slow rate of adenylate cyclase activation is evident in Figures 3(A) and 3(B). Within the usual time scale chosen in many experimental studies, this activity can easily elude notice, especially when the experiment is performed with relatively low concentrations of Gpp(NH)p (less than 10 μ M) in the presence of GDP, without preincubating the membranes with the nucleotide. The time scale of this activation is consistent with the recently reported rates of GTP[S] binding to wild-type or to different mutants of G $_i$ α_1 [33]. The complex appearance of the adenylate cyclase activation kinetics (Figure 3B) was closely related to the presence of added GDP in the incubation buffer. With no GDP added, the activation

pattern was apparently exponential (Figure 3A). In the present study we did not investigate further the details of this activation kinetics. For present purposes, however, it is more critical to note the dependence of the steady-state cyclase activity on the concentration of Gpp(NH)p. Fitting a simple exponential growth to the data in Figure 3(A) provides diverging estimates of the steady state at various concentrations of Gpp(NH)p (as assessed by using a t test in which asymptotic standard errors of the estimates were used). The same pattern is also evident on inspection of Figure 3(B). In this case, however, it is more difficult to make a quantitative assessment owing to the complex kinetics, of unknown origin, in cyclase activation. At this point we used an empirical logistic equation (see the Materials and methods section) to fit the data in Figure 3(B). Although the choice of the regression model was quite arbitrary, it gave virtually perfect fits and the asymptotes thus estimated were consistent with the results of independent experiments in two respects: (1) when we plotted the asymptotes that were estimated from Figures 3(A) and 3(B) against the concentration of Gpp(NH)p, the resulting picture was consistent with an equilibrium competition behaviour that might take place between GDP and Gpp(NH)p (see Figure 3D), and (2) estimated EC $_{50}$ values for Gpp(NH)p from the data in Figure 3(D) were in perfect agreement with those estimated from a set of experiments in which a completely different strategy was followed (see Figure 7 and related discussion below). Thus the addition of GDP to the incubation medium seems to shift the steady-state activation curves rightwards. We showed these results in Figure 3(D) by plotting the asymptotes estimated from the activation curves in Figures 3(A) and 3(B) against the concentration of Gpp(NH)p. This relationship can be predicted from the framework of the quaternary complex model, assuming that the nucleotide binds to G α reversibly to form an equilibrium complex, the amount of which depends on the concentration of the nucleotide.

Figure 3(C) shows how the results of these experiments are predicted by the collision coupling model. In this simulation we set the encounter frequency (ω) and mean duration of encounters (τ) as 45 min $^{-1}$ and 1.5 min respectively, as suggested by Stickle and Barber for the β -adrenoceptor system in S49 lymphoma cells [16]. We adjusted the k_e value in such a way that the apparent rates of activation (k_a) fell in the range of minimum (0.005 ± 0.0009 min $^{-1}$) and maximum (0.02 ± 0.002 min $^{-1}$) values ($\pm$ S.E.M.) estimated from Figure 3(A) for the lowest and highest concentrations of Gpp(NH)p respectively (see Figure 2 for the definition of the parameters). This procedure yields a range of k_e values between 3 and 11 s $^{-1}$, which incidentally covers the value (6.9 s $^{-1}$) that was used by Stickle and Barber in S49 cells. The agreement between these values is loosely connected to the choice of k_{on} and k_{off} . More importantly, however, the simulation in Figure 3(C) shows that the fractional activation of adenylate cyclase will always be 1 at steady state. This result is also evident analytically when we consider the fractional steady-state activation (f) predicted by the encounter coupling model: $f = k_a / (k_a + k_i)$, where k_i is the inactivation rate. According to this relationship f will always be 1 for all non-zero values of k_a , when $k_i = 0$. Therefore the commonly made assumption in using the collision coupling model that the activation is irreversible in the absence of GTPase ($k_i = 0$), does not apply to Gpp(NH)p in turkey erythrocyte membranes.

Effects of propranolol on the adenylate cyclase activated by isoprenaline in the presence of GTP or Gpp(NH)p

The observation that the steady-state activity of adenylate cyclase depends on the concentration of Gpp(NH)p might not necessarily

Figure 3 Time- and concentration-dependent activation of adenylate cyclase in turkey erythrocyte membranes in the presence of Gpp(NH)p

(A) Fractional adenylate cyclase activities determined at the indicated time points from the incubations with Gpp(NH)p at the indicated concentrations. Data are shown as means $\pm$ S.E.M. ($n = 3$). Solid curves are the best fit of a simple exponential growth. Data are normalized with respect to the maximal cyclase activity obtained at 100 μ M Gpp(NH)p (120–180 pmol/min per mg of protein). Estimated asymptotes for [Gpp(NH)p] = 100, 1, 0.1 and 0.01 (μ M) are 1.02 ± 0.04 , 1.03 ± 0.06 , 0.67 ± 0.02 and 0.18 ± 0.06 respectively. (B) As in (A) but GDP and UDP (1 μ M and 1 mM respectively) were added to the incubation medium. Solid curves are the best fit of the empirical logistic equation $y = a - a/[1 + e^{-b(t-a)}]^{d_1}$. Data are normalized with respect to the maximal cyclase activity obtained at 100 μ M Gpp(NH)p (120–180 pmol/min per mg of protein). Estimated asymptotes for [Gpp(NH)p] = 100, 10, 1, 0.1 and 0.01 (μ M) are 0.99 ± 0.02 , 1.00 ± 0.01 , 0.83 ± 0.02 , 0.41 ± 0.02 and 0.21 ± 0.1 respectively. (C) Prediction of the encounter coupling model for the results of the experiments shown in (A). To simulate different concentrations of Gpp(NH)p, the efficiency parameter, k_0 , was manipulated as explained in the text. The curves obtained with the rate constants estimated for the lowest and highest concentrations of the guanine nucleotide used are indicated as L and H respectively (see text for details). Inactivation rate for G-protein, k_1 is zero. (D) The estimated steady states (asymptotes) from the data given in (A) and (B) are plotted against $\log[\text{Gpp(NH)p}]$.

indicate the reversibility of nucleotide action in the absence of GTPase. It can be argued that the heterogeneous abundance of different G-proteins such as G_s and G_i , which have opposite actions on adenylate cyclase in the membranes, might lead to such observations. In other words, Gpp(NH)p might activate G_i and G_s with different affinities and 'efficacies', which might result in variable contributions of these components to the observed adenylate cyclase activity at different concentrations of Gpp(NH)p. This situation might lead to the above observation even when the action of Gpp(NH)p is irreversible, if saturation of G_i by Gpp(NH)p occurs before that of G_s . To clarify this point we investigated the reversibility of isoprenaline-induced adenylate cyclase activity by using propranolol in the presence of Gpp(NH)p (or GTP). We assumed that isoprenaline-induced activation is specifically mediated by G_s .

We incubated the membranes with isoprenaline and GTP or Gpp(NH)p in the presence or absence of propranolol, and measured the adenylate cyclase activity at the indicated time points. Propranolol was added at either the beginning or the middle of the incubation period. These two experiments, together with that using the membranes that did not receive propranolol, were run simultaneously and presented in the same Figure. When GTP was used, the activation of adenylate cyclase was immeasurably fast and the activity decayed slowly with time, probably owing to specific or non-specific GTPases (Figure 4A). Thermal inactivation of adenylate cyclase or G_s during the incubation as a mechanism for this decay seems to be unlikely, because such a decay did not occur when Gpp(NH)p was used instead of GTP (not shown, but see also the adenylate cyclase activity after the addition of propranolol in Figure 4B). The

Figure 4 Effect of propranolol (1 μ M) on isoprenaline (1 μ M)-activated adenylyl cyclase in the presence of 10 μ M GTP (A) or 10 nM Gpp(NH)p (B) in turkey erythrocyte membranes

Incubations started at $t = 0$, with nucleotide plus isoprenaline (●), nucleotide plus isoprenaline then propranolol at the indicated time (■), and nucleotide plus isoprenaline plus propranolol (▲). Data are shown as means $\pm$ S.E.M. ($n = 3$). Continuous curves are arbitrary interpolations of the observed values with multiple exponential. The dotted curve interpolates the trend after the addition of propranolol. The dotted line in (B) shows the prediction of the collision or the encounter coupling model (CCM) after the addition of propranolol. Extrapolating the trend of the lowermost curve in (B) reaches the value obtained after the addition of propranolol (result not shown).

addition of propranolol inhibited the agonist-induced activity when GTP was used (Figure 4A), which is consistent with the presence of intrinsic GTPase of $G_s\alpha$. According to the collision coupling model, the outcome of this experiment would be totally different if Gpp(NH)p were used instead of GTP: adenylyl cyclase would keep its activity after the addition of propranolol. Experiments showed that this is not the case. Figure 4(B) shows the results of such an experiment. In the absence of GTPase, propranolol 'reversed' the agonist-induced adenylyl cyclase activity to a level that was achieved with nucleotide alone. This pattern was also valid at different concentrations of Gpp(NH)p (see below). This result not only shows the reversibility of activation in the absence of GTPase but also invalidates one of the main assumptions of the collision coupling idea, namely that once activated, the state of G-protein is independent of the receptor's state. In other words, it is evident that active (agonist-bound) receptors should keep signalling to the active G-proteins

Figure 5 Concentration of GDP relative to its initial value (25 μ M) during the incubation with turkey erythrocyte membranes (15 μ g/100 μ l) at 37 $^{\circ}$ C, in the absence (○) or presence (●) of UDP (1 mM)

to retain them in their active state even in the absence of a GTPase-mediated inactivation. Hence, the inactivation of receptors by propranolol results in a relaxation of the whole system.

Predicting that this reversal might be due to an inverse nucleotide exchange (from Gpp(NH)p to GDP), we included GDP in all these experiments to make the reversal observable. However, the addition of GDP into the incubation buffers raises the possibility of GTP contamination that can arise from conversion of GDP to GTP during such long incubation periods [34]. The contribution of the contaminating GTP to the agonist-induced activation and its hydrolysis, which becomes apparent after the receptor's inactivation, might account for the above observations. We eliminated this possibility by analysing the nucleotide composition during incubation by using an HPLC system. In our experimental system, GDP disappeared slowly by conversion to GMP rather than to GTP. This conversion was substantially blocked by UDP (Figure 5), which we added to the incubation buffers whenever GDP was present. This analysis also showed that Gpp(NH)p stays stable within the time scale of the experiments and that the initial contamination by GTP in the Gpp(NH)p preparations is too small to explain the observed reversal of adenylyl cyclase activity on the addition of propranolol.

Dependence of agonist-induced net reversible activation of adenylyl cyclase on the concentration of Gpp(NH)p

Under certain circumstances, the quaternary complex model predicts that the agonist-induced net activation disappears at relatively high concentrations of the nucleotide (Figure 6). In other words, propranolol-induced inactivation of agonist-stimulated adenylyl cyclase (see Figure 4B) is expected to vanish in some cases as the concentration of non-hydrolysable nucleotide increases. This prediction is based on two assumptions: (1) the 'only' final action of agonist that contributes to the activation process is to provide a nucleotide exchange on $G\alpha$, and (2) Gpp(NH)p opposes the binding of $G_s\beta\gamma$ to $G_s\alpha$ so strongly that virtually no heterotrimer can exist at equilibrium when $G\alpha$ is saturated by the nucleotide. The implication of these two assumptions is that Gpp(NH)p-saturated $G\alpha$ is fully active.

Figure 6 Computer simulation of G-protein activation with the use of the equilibrium quinary complex model depending on the concentration of activating guanine nucleotide

Curves a and b are obtained in the saturating presence or in the absence of agonist stimulation, and the curves are normalized with respect to the value obtained in the saturating presence of agonist and nucleotide. Activation is considered as the total concentration of GTP-bound and $G\beta\gamma$ -free species. Inset, net activation induced by agonist (curve a minus curve b) depending on the guanine nucleotide concentration. Concentrations of protein species are in the order of their affinity values used, and the rank order of their total concentrations are receptor $< G\alpha < G\beta\gamma$. Unconditional affinities are set as $K = 10^7$, $M = 10^{10}$, $L = 10^{10}$, $J_{GDP} = 10^7$, $J_{GTP} = 10^7$ (M^{-1}). Values for coupling factors are as follows: $\alpha = 200$, $\beta_{GTP} = 0.1$, $\beta_{GDP} = 0.5$, $\phi_{GTP} = 0.001$, $\phi_{GDP} = 10$, $\delta = 0.01$, and all the higher order interactions are set to 1. These coupling factors (α , β , ϕ , δ) describe the allosteric interactions between ligand and $G\alpha$, ligand and nucleotide, nucleotide and $G\beta\gamma$, and $G\beta\gamma$ and receptor respectively (see also Figure 1). Total concentration of GDP (as a contaminant) was assumed to be equal to that of $G\alpha$.

Consequently, agonist cannot induce any additional activation of G-protein when $G\alpha$ is fully saturated by Gpp(NH)p. This model would also explain why at steady state the agonist-induced activation in the absence of GTPase seemed irreversible (because the steady-state activity should be the same regardless of the presence of agonist), or equivalently, why agonist cannot further activate the adenylate cyclase when a saturating concentration of Gpp(NH)p is used, as in the early experiments that led to the collision coupling idea. Accordingly the only action of agonist should be to accelerate the rate of activation, which eventually reaches a unique equilibrium that is determined by saturating nucleotide. To evaluate this possibility and to provide more insight into the mechanism of the above-mentioned phenomena (Figures 3 and 4), we investigated the agonist induced reversible activation of adenylate cyclase over a wide range of Gpp(NH)p concentrations (0–100 μ M).

We incubated the membranes at the indicated concentrations of Gpp(NH)p in the presence of isoprenaline (1 μ M) for 3 h, which was long enough to reach equilibrium in the presence of agonist. At the end of this incubation period, some of the membranes received propranolol. We assayed adenylate cyclase activity after another 15 min of incubation with or without propranolol. A representative example for such an experiment is given in Figure 7. The results shown in Figure 7 confirm that in the presence (or absence) of isoprenaline, the steady-state activation of adenylate cyclase depends on the concentration of Gpp(NH)p (compare Figures 7 and 3). The general pattern of the lower curve in Figure 7(A), indicated by 'propranolol (+)', is consistent with the estimated asymptotes from the data given in Figure 3. There is no significant difference between the EC_{50}

Figure 7 Dependence of steady-state adenylate cyclase activity on the concentration of Gpp(NH)p in turkey erythrocyte membranes

(A) Adenylate cyclase activity measured after 195 min of incubation (37 °C) in the presence of isoprenaline (1 μ M), GDP + UDP (1 μ M–1 mM) and indicated concentrations of Gpp(NH)p. At the final 15 min of incubation, membranes received either 10 μ l of propranolol (1 μ M final) or water, indicated as propranolol(+) and propranolol(–) respectively. Data are shown as means $\pm$ S.E.M. ($n = 3$). Fitted curves are four-parameter logistics. (B) Isoprenaline-induced reversible activation calculated as the difference between the two curves in (A). (C) As in (A), but (+)-propranolol was used instead of its (–) isomer to test a putative non-specific effect of propranolol. There is no significant effect of (+)-propranolol on isoprenaline-activated adenylate cyclase.

values for Gpp(NH)p that were estimated from Figure 3(D) (the right-hand curve, $\log[EC_{50}] = 6.6 \pm 0.07$) and those taken from the curve labelled 'propranolol(+)' in Figure 7(A) ($\log[EC_{50}] = 6.5 \pm 0.05$). The control experiments shown in Figure 7(C) that we made with (+)-propranolol indicate that the observed effect of propranolol in figure 7(A) (as well as in Figure 4) is due to its receptor-blocking effect, rather than to its membrane-disturbing

Figure 8 The effect of forskolin on the competition binding of isoprenaline in the presence (left panel) or absence (right panel) of Gpp(NH)p ($1 \mu\text{M}$) in turkey erythrocyte membranes

The two binding curves shown in the right panel superimpose perfectly. Results are mean values of three experiments.

properties. Therefore the lower curve in Figure 7(A) can be considered as equivalent to the cyclase activity that would be obtained with Gpp(NH)p alone. However, contrary to our expectations, based on the above discussion, that the net cyclase activity should vanish at high concentrations of Gpp(NH)p, the agonist-induced steady-state adenylate cyclase activity increased with concentration of Gpp(NH)p. Regarding the design of these experiments, agonist-induced activation can be considered reversible over a wide range of nucleotide concentrations. This pattern was independent of the presence of added GDP. The only effect of GDP (from 0 to $10 \mu\text{M}$) was to shift the activation curves rightwards (results not shown, but see also Figure 3D). The EC_{50} of nucleotide-induced activation curves in the presence of 0, 1 and $10 \mu\text{M}$ GDP were 0.3 ± 0.1 , 0.5 ± 0.07 and $0.9 \pm 0.09 \mu\text{M}$ respectively. Although these observations clearly show that the activation induced by isoprenaline is reversible in the absence of GTPase, the mechanism of agonist-induced activation, especially at high concentrations of Gpp(NH)p, is not obvious. This is because, considering the nanomolar dissociation constants for guanine nucleotide- $\text{G}\alpha$ complexes [2], G_s should be fully saturated at relatively high concentrations of Gpp(NH)p. Thus nucleotide exchange on $\text{G}\alpha$, which is already saturated at micromolar concentrations of Gpp(NH)p, cannot account for the observed agonist-induced adenylate cyclase activation. The curves in Figure 7 suggest that isoprenaline-induced activation does not vanish with increasing concentrations of the nucleotide. We considered two possibilities at this point: (1) agonist might directly increase the interaction between the Gpp(NH)p- $\text{G}\alpha$ complex and adenylate cyclase, or (2) a 'residual' Gpp(NH)p-bound heterotrimer (supposed to be inactive), which is in 'contact' with the receptor, can be further destabilized to induce an additional activation of the effector on binding of agonist to the receptor. The first possibility requires that agonist (H), receptor (R), $\text{G}_s\alpha$ (G), guanine nucleotide (N) and adenylate cyclase (C) form an equilibrium complex, HRGNC. If the equilibrium association of adenylate cyclase to HRGN is more efficient than that to RGN, then agonist can induce additional $\text{G}_s\alpha$ -adenylate cyclase association (and activation) even when $\text{G}_s\alpha$ is fully saturated with guanine nucleotide. The second possibility implies that saturation of G_s with Gpp(NH)p cannot fully dissociate the G_s into its subunits. These possibilities are relevant only when the effector (adenylate cyclase) is not limiting, which is obviously the case in the present experimental system.

We used an indirect experimental approach to evaluate the first possibility. Available evidence suggests that there must be some equilibrium interactions between H, R, G and C (at least in

certain experimental systems): (1) it is known that the binding efficiency of forskolin to adenylate cyclase or the ability of forskolin to stimulate this enzyme is increased by the presence of G_s [35]; (2) isoprenaline increases the binding of forskolin to adenylate cyclase [36] and vice versa [37]; and (3) overexpression of β -AR on Sf9 cells increases the efficiency of forskolin in stimulating adenylate cyclase [38]. If all these species form an equilibrium complex (HRG(N)C-forskolin), then thermodynamic considerations imply that as long as the binding of forskolin is linked to that of agonist, the binding of agonist should also be linked to that of forskolin, as has also been shown experimentally [point (2) above]. Using this experimental paradigm, we tested the effect of forskolin on the binding of isoprenaline in the absence or presence of Gpp(NH)p. Results (Figure 8) showed no obvious linkage between forskolin and isoprenaline. Although there was a slight effect of forskolin on the isoprenaline binding curve in the presence of Gpp(NH)p (Figure 8, left panel), it was in the opposite direction. Forskolin had no effect at all on the binding of [^{125}I]iodocyanopindolol. In this case it seems unlikely that isoprenaline increases the equilibrium binding of $\text{G}_s\alpha$ to adenylate cyclase, at least in the present experimental system. Thus it is also unlikely that isoprenaline induces adenylate cyclase activation in the Gpp(NH)p-saturated system by remotely increasing the $\text{G}_s\alpha$ -adenylate cyclase interaction. In contrast, the hypothesis of residual heterotrimer that can be further destabilized by isoprenaline finds a sound explanation in the framework of the quaternary complex model. Using the quaternary complex model, we simulated the results of the experiment given in Figure 7. In this simulation (Figure 9), the coupling factor describing the effect of each of Gpp(NH)p and $\text{G}\beta\gamma$ on the binding of the other when they are simultaneously bound to $\text{G}\alpha$ ($\phi_{\text{Gpp(NH)p}}$) was set to 0.9, which allows the formation of a residual heterotrimer when $\text{G}\alpha$ is fully saturated by Gpp(NH)p. All other parameter values are the same as in the legend of Figure 6. Nucleotide-dependent activation (with or without agonist stimulation) is shown in Figure 9(A). We also showed the relative concentrations of nucleotide-bound and $\text{G}\beta\gamma$ -bound forms of $\text{G}\alpha$ in Figure 9(B). It is evident in Figure 9(B) that agonist can decrease the concentration of residual heterotrimer without increasing the nucleotide binding, which is already saturated by a high concentration of the nucleotide. Activation in Figure 9(A) is given as the total concentration of Gpp(NH)p-bound and $\text{G}\beta\gamma$ -free species. If, however, we include the concentration of free $\text{G}\beta\gamma$ in the active species with a certain weight, assuming that the free $\text{G}\beta\gamma$ (released from the heterotrimer) also has some stimulating effect on adenylate cyclase,

Figure 9 Computer simulation of the experimental results given in Figure 7 with the use of the quinternary complex model

(A) Curves represent the nucleotide-induced fractional activation in the presence or absence of saturating agonist stimulation. The parameter values are identical with those given in the legend to Figure 6, except that $\phi_{\text{GTP}} = 0.9$. Activation is considered as the total concentration of GTP-bound and $G\beta\gamma$ -free species, and normalized with respect to the value obtained in the saturating presence of agonist and nucleotide. (B) Dependence on the agonist concentration of the concentration of $G\alpha$ that is bound with nucleotide (GTP-bound species) or $G\beta\gamma$ (Heterotrimer). Concentrations are given on a normalized scale with respect to the total $G\alpha$ and total heterotrimer at zero concentration of agonist respectively. Concentration of Gpp(NH)p was set at 1 mM. (C) As in (A), but free $G\beta\gamma$ is also included in the active species. Weight of $G\beta\gamma$ in the active species was assumed to be 20% of the $G\alpha$ -containing (active) species. This is equivalent to assuming that the relative abundance of $G\beta\gamma$ -sensitive adenylate cyclase is 20% of the total cyclase population. (D) Net activation calculated from the curves in (C).

Figure 10 Competition binding curves for isoprenaline obtained in the absence or presence of Gpp(NH)p (1 μ M) (A) and computer simulation of the same experiment with the use of the quinternary complex model with the parameter values given in the legend to Figure 9(B)

Results are means of three experiments.

then the model predicts the experimental results even more precisely [compare Figures 9(C) and 9(D) with Figures 7(A) and 7(B)]. Interestingly, this model, with the parameters used to simulate adenylate cyclase activation, also predicts the well-known lack of guanine nucleotide effect on agonist binding in turkey erythrocyte membranes. Figure 10 shows the theoretical and experimental results for the effect of Gpp(NH)p on the binding of isoprenaline. Although this result does not imply any

causal relationship between the two phenomena (activation and agonist-binding patterns), it provides a simultaneous explanation for the two seemingly independent events. On an intuitive basis, when the average result of agonist action in a particular instance of parameter values is to oppose the heterotrimer formation directly rather than indirectly by mediating nucleotide exchange, a lack of nucleotide effect on agonist binding should not be surprising in this instance.

DISCUSSION

In this study we investigated the equilibrium coupling of β -AR to adenylate cyclase in turkey erythrocyte membranes, with particular reference to theoretical models and their assumptions that describe this coupling. We showed that: (1) the collision (or encounter) coupling model in its present form is not a suitable approach for 'understanding' the mechanism of coupling between β -AR and G_s -adenylate cyclase, although it mimics certain non-equilibrium behaviours of this system; (2) steady-state coupling of β -AR to adenylate cyclase can be successfully explained by using a quinternary complex model based on allosteric equilibria that occur between the components of the signalling system (i.e. receptor, subunits of G-protein and their ligands); (3) agonist-induced activation of the guanine nucleotide-saturated system requires a mechanism other than the nucleotide exchange on $G\alpha$. This mechanism can be expected when the complex allosteric interactions between the components of the signalling system are taken into account. To the best of our knowledge, we provide here for the first time equilibrium data for the coupling of β -AR and G_s -adenylate cyclase and evidence for a direct negative interaction between receptor and $G\beta\gamma$ on $G\alpha$ in turkey erythrocyte membranes. These points and their implications are briefly discussed below.

Role of receptor in the activation of G-protein

A great deal is known about the structural and functional properties of G-proteins and associated receptors, owing to recent molecular biological and crystallographic studies [33,39–43]. However, we still lack detailed molecular information for predicting quantitatively the mode of interaction between receptor and the subunits of G-protein. Therefore this sort of information is still deduced from indirect experimental data that exemplify certain instances of the entire functional repertoire of the G-protein-mediated signalling systems. At this point, theoretical models constitute an indispensable tool for making these data intelligible and for constructing logical links between different observations. Two different approaches, namely the collision coupling and ternary complex types of model, seem to fulfil this requirement in the field. However, the way in which these models describe the role of the receptor in regulating the activity of the other components leads to entirely different pictures of receptor action. This diversity is summarized in a very simple scheme in Figure 11. The implications of these ideas are beyond the mere choice of a schematic representation. In collision

Figure 11 Schematic representation of the concept of receptor action in the collision coupling and allosteric equilibrium models

R, G and G^* signify active receptor, inactive and active G-proteins respectively. The dotted path stands for the receptor-independent inactivation (GTPase). Rate constants for receptor-induced activation of G-protein and its inactivation (k_a and k_i respectively) are also indicated for the collision coupling model (see also Figure 2). The overall equilibrium constants, which govern the allosteric scheme, depend (in a complicated way) on the parameters that are described in the legend to Figure 1.

coupling, activated G-protein stops 'feeling' the state of the receptor and its future is completely determined by an independent path of inactivation, whereas in the other case the receptor keeps contributing to the activation of G-protein by shifting allosterically the G-protein equilibrium towards the active state of the G-protein, thus 'stabilizing' the G-protein in its active state. The origin of this diversity is not actually the collisional mechanism itself but the commonly made assumptions in the 'collision coupling models' that activation and inactivation are independent and irreversible events. An inevitable consequence of this view becomes apparent when we consider the system in the absence of GTPase; terminating the receptor action does not affect the state of the activated G-proteins in the first case, and restores the unstimulated state of the system in the latter. Experiments showed that the latter outcome is the case (Figures 4B and 7) for the β -AR- G_s -adenylate cyclase system of turkey erythrocyte membranes, the operation of which has long been considered otherwise. Therefore we conclude that the β -AR in the turkey erythrocyte membranes cannot be a steady-state catalyst in the 'collision coupling' sense, and suggest that the β -AR action can be analysed by assuming the receptor to be an allosteric ligand for G-protein that can be best understood and measured at thermodynamic equilibrium. Considering that the guanine nucleotides dissociate very slowly from $G\alpha$ in the presence of Mg^{2+} [44,45], the observed fast reversal of agonist-induced adenylate cyclase activity by propranolol can hardly be attributed to the dissociation of Gpp(NH)p from $G\alpha$ on inactivation of the receptor. We were unable to show such a dissociation by measuring directly the agonist-induced specific binding to, or antagonist-induced release of, Gpp(NH)p from $G\alpha$, owing to a low signal-to-noise ratio in this experimental system. None the less, dissociation of Gpp(NH)p alone (even if it occurs), resulting in an 'empty' or GDP-bound $G\alpha$, in the absence or presence of GDP respectively, cannot account for the present data obtained at saturating concentrations of Gpp(NH)p. The data in Figure 7 strongly suggest an additional role for the receptor (and agonist) in activating the G-protein (and adenylate cyclase) when the concentration of Gpp(NH)p is saturating.

Heterotropic interactions on $G\alpha$

Another interesting point is the nature of activation of G-protein by the receptor. It is very well documented that the exchange of GTP (or its analogues) with GDP on $G\alpha$ and dissociation of G-protein into its α and $\beta\gamma$ subunits are sufficient and necessary conditions for G-proteins to modulate the effector's activity [1]. However, there are no available experimental data that might directly show us how the receptor provides these requirements to control the activity of G-protein. Does receptor protein primarily induce a nucleotide exchange (dissociation of GDP and binding of GTP), which then leads to subunit dissociation owing to the negative allosteric coupling between GTP and $G\beta\gamma$ [46], does it primarily loosen the association of heterotrimer, which eventually results in nucleotide exchange for the same reason, or does it have both actions? The only evidence for resolving this circle is the ability of the receptor to facilitate the nucleotide exchange (but only in the presence of $G\beta\gamma$ [47–49]) and the negative allosteric coupling between the binding of GTP and $G\beta\gamma$ to $G\alpha$. This information is obviously insufficient to answer the question. The missing part of the circle is an experiment that should tell us the effect of the receptor on the heterotrimer stability independently of the nucleotide. However, such an experiment seems to be technically problematic. A theoretical discussion of these circular heterotropic interactions that might take place between $G\beta\gamma$, guanine nucleotides and receptor on $G\alpha$ is available [6]. In

this theoretical treatment it was assumed that heterotropic allosteric interaction between the receptor and $G\beta\gamma$ is negative. Here we provide still indirect but suggestive experimental evidence for the existence of such a negative heterotropic effect: if we assume that the role of the receptor in the activation of G-protein is to provide a nucleotide exchange on $G\alpha$ directly without having any direct effect on the heterotrimer stability, then it seems impossible to explain the data in Figure 7. In that experiment, isoprenaline still induced a pronounced reversible activation of adenylate cyclase even in the presence of 100 μ M Gpp(NH)p. This concentration for the guanine nucleotide is high enough to saturate $G\alpha$. Thus if the only action of the receptor were to induce a nucleotide exchange, we would observe no agonist effect when $G\alpha$ is already in Gpp(NH)p bound form. However, if the conditional equilibrium

exists, as pointed out by Brandt and Ross [50], then the concentrations of these species would depend on the concentrations of G-protein subunits in the membrane and on the equilibrium constant for this reaction when Gpp(NH)p is saturating. The left-hand side of this equilibrium forms the active species. Consequently, in an experimental system where the concentrations of the subunits are constant, a negative heterotropic effect of the receptor on the binding of $G\beta\gamma$ to $G\alpha$ (favouring the above reaction towards the left) perfectly explains the ability of agonist to activate the nucleotide-saturated G-protein and adenylate cyclase. The recently published crystal structure of heterotrimeric transducin also supports such a mechanism [51]. The refined crystal structure of the heterotrimer, together with the mutational data, suggests that the receptor can interact with helices at the N- and C-termini of $G\alpha$. The former site is closely related to one of the contact regions between $G\alpha$ and $G\beta\gamma$, whereas the latter seems to be closer to the switch regions (which also form an interface between $G\alpha$ and $G\beta\gamma$) and to the nucleotide-binding pocket on $G\alpha$. Thus the receptor might affect the association of heterotrimer by modifying the switch region (via nucleotide exchange) and/or modifying the N-terminal helix of $G\alpha$. Consequently it seems quite possible that GTP-bound $G\alpha$ can still be in contact with $G\beta\gamma$ via its N-terminal helix (α N), and that this contact can be further loosened by the interaction of activated receptor with this region. Such a mechanism is also consistent with the relatively fast inactivation of isoprenaline-induced adenylate cyclase activity by propranolol observed in the present experiments. One would expect an inactivation due to a $G\beta\gamma$ -independent dissociation of Gpp(NH)p to be much slower than the observed rate (Figure 4B) [45,52]. This model is independent of whether the adenylate cyclase in our experimental system is activated by $G\alpha$ or $G\beta\gamma$ [53]. Nevertheless the present simulations show that the inclusion of $G\beta\gamma$ in the active species provides a better fit to the experimental data (compare Figures 9D and 9A).

Simulations with the quinternary complex model according to the above scenario suggest that the extent of agonist-induced activation in high nucleotide concentrations should be very sensitive to the relative concentration of $G\beta\gamma$ in the membrane (results not shown). Thus such a prediction can be tested in a transfection experiment in which the agonist is expected to induce higher net activation in the clones that overexpress $G\beta\gamma$. Smaller activation, in contrast, is expected with the overexpression of a $G\alpha$ (such as transducin) that does not interact with adenylate cyclase but binds $G\beta\gamma$ to reduce its effective concentration in the cell membrane. Dependence of reversible agonist action on the relative concentration of $G\beta\gamma$ at high concentrations of guanine nucleotide would also explain the

variable observation of this phenomenon in different systems under different conditions, considering the fact that the relative expression of $G\beta\gamma$ can vary between different systems or between different states of the same system [54].

A careful look at the data in Figure 7 shows that isoprenaline can activate the adenylate cyclase consistently at low concentrations, or even in the absence of Gpp(NH)p. Considering the design of the experiments, this observation cannot be explained by contamination with GTP. The membranes were incubated with isoprenaline for 3 h, which was long enough for the stimulated G_s to hydrolyse the bound GTP. Although we did not measure the agonist-stimulated rate of GTPase directly, Figure 4(A) suggests that this activity is present and fast compared with the time scale of the incubations. Another unlikely explanation would be the agonist-induced dissociation of GDP from $G\alpha$, resulting in an empty form of G-protein that could be active. In that case, this activation should have been sensitive to the concentration of GDP, but it was not. In contrast, the explanation given for the agonist-induced activation at high concentrations of nucleotide also provides a reasonable speculation for the observation we are considering. If there is a certain abundance of the $G\beta\gamma$ -sensitive isotypes of adenylate cyclase (type II and/or IV) in the membranes, then agonist-induced dissociation of a small amount of heterotrimer (into GDP- $G\alpha$ and $G\beta\gamma$) may activate adenylate cyclase (Figure 9D). Nevertheless we have no evidence for such an abundance.

Although it is obvious from the present results that an additional effect of the receptor should be considered in agonist-induced activation at high concentrations of the nucleotide in turkey erythrocyte membranes, we do not know how much this and direct 'nucleotide-exchanger' effects of the receptor contribute to the final action of agonist at intermediate concentrations of Gpp(NH)p.

In conclusion, complex interactions between the components of the β -AR- G_s -adenylate cyclase system of turkey erythrocyte membranes can be best understood when we consider the heterotropic allosteric interactions at thermodynamic equilibrium. The current form of the collision (or encounter) coupling model leads to a confusing concept of receptor action that seems to be an inappropriate approximation to the present experimental data because, unlike the nucleotide exchanger effect of the receptor, the direct destabilizing effect of receptor on the heterotrimer requires a continuous interaction between the active receptor and $G\alpha$ to keep the $G\alpha$ in a dissociated form, which is consistent with the fast reversal by propranolol of the cyclase activity induced by isoprenaline at saturating Gpp(NH)p concentrations. For the sake of conceptual consistency, the collision coupling model can be modified to incorporate these allosteric interactions by considering the binding reactions to be reversible in a strict thermodynamic sense. Such a manipulation is actually equivalent to dropping the simple assumptions of the collision coupling model that the activation and inactivation are irreversible and independent events (which we call the 'irreversibility and independence assumption'). With this assumption dropped, the collision coupling model becomes perfectly compatible with the ideas behind the ternary complex type of equilibrium models. In this case the collision coupling model can be seen as a specific kinetic interpretation of the equilibrium models. In other words, without the 'irreversibility and independence assumption', the only implication of the model will simply be that the association rate constant for the formation of receptor-G-protein complex is determined by lateral diffusion-based collisional events. Such a mechanism is neither excluded nor specified in the equilibrium models, and thus does not contradict the equilibrium approaches. However, the 'irreversibility and

independence assumption' is a necessary requirement for the collision coupling model to accomplish a steady-state signal amplification at the G-protein level, for which the model was actually intended. Hence dropping this assumption will disable the collision coupling model to predict a steady-state stoichiometric signal amplification. None the less, such a steady-state catalytic view of the receptor, i.e. that which specifically contradicts the equilibrium models, did not meet the experimental data obtained in the present experimental system. It can be argued at this point that thermodynamic equilibrium itself is incompatible with life. This fact constitutes a good reason for bringing the ternary complex type of models into a kinetic analysis once the nature of allosteric interactions is completely understood. High-resolution experimental data for the kinetics of the ternary complex scheme have been reported recently for the formyl peptide receptor system in neutrophil membranes [55,56]. Such an analysis, also taking into consideration the GTPase reaction, would provide a consistent and more complete theory of G-protein-mediated signal transduction.

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REFERENCES

- Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56**, 615–649
- Bourne, H. R., Sanders, D. A. and McCormick, F. (1991) *Nature (London)* **349**, 117–127
- Clapham, D. E. and Neer, E. J. (1993) *Nature (London)* **365**, 403–406
- DeLean, A., Stadel, J. M. and Lefkowitz, R. J. (1980) *J. Biol. Chem.* **255**, 7108–7117
- Ehlert, F. J. and Rathbun, B. E. (1990) *Mol. Pharmacol.* **38**, 148–158
- Onaran, H. O., Costa, T. and Rodbard, D. (1993) *Mol. Pharmacol.* **43**, 245–256
- Samama, P., Cotecchia, S., Costa, T. and Lefkowitz, R. J. (1993) *J. Biol. Chem.* **268**, 4625–4636
- Weber, G. (1972) *Biochemistry* **11**, 864–878
- Ehlert, F. J. (1985) *Mol. Pharmacol.* **28**, 410–421
- Costa, T., Ogino, Y., Munson, P. J., Onaran, H. O. and Rodbard, D. (1992) *Mol. Pharmacol.* **41**, 549–560
- Tolkowski, A. M. and Levitzki, A. (1978) *Biochemistry* **17**, 3795–3810
- Levitzki, A. (1986) *Physiol. Rev.* **66**, 819–854
- Bergman, R. N. and Hechter, O. (1978) *J. Biol. Chem.* **253**, 3238–3250
- Gross, W. and Lohse, M. J. (1991) *Mol. Pharmacol.* **39**, 524–530
- Stickley, D. and Barber, R. (1992) *Biochem. Pharmacol.* **43**, 2015–2028
- Stickley, D. and Barber, R. (1993) *Mol. Pharmacol.* **43**, 397–411
- Ross, E. M., Maguire, M. E., Sturgill, T. W., Biltonen, R. L. and Gilman, A. G. (1977) *J. Biol. Chem.* **252**, 5761–5775
- Cheung, A. H., Huang, R. C., Graziano, M. P. and Strader, C. P. (1991) *FEBS Lett.* **279**, 277–280
- Senogles, S. E., Benovic, J. E., Amlaiky, N., Unson, C., Milligan, G., Vinitzky, R., Spiegel, A. M. and Caron, M. G. (1987) *J. Biol. Chem.* **262**, 4860–4867
- Murray, R. and Keenan, A. K. (1989) *Cell. Signal.* **1**, 173–179
- Fawzi, A. B. and Northup, J. K. (1990) *Biochemistry* **29**, 3804–3812
- Okamoto, T., Murayama, Y., Hayashi, Y., Inagaki, M., Ogata, E. and Nishimoto, I. (1991) *Cell* **67**, 723–730
- Cussac, D., Kordon, C., Engalbert, A. and Saltarelli, D. (1993) *Cell. Signal.* **5**, 119–137
- Cassel, D. and Selinger, Z. (1977) *J. Cyclic Nucl. Res.* **3**, 11–22
- Lefkowitz, R. J., Stadel, J. M. and Caron, M. G. (1983) *Annu. Rev. Biochem.* **52**, 159–186
- Stadel, J. M., DeLéan, A. and Lefkowitz, R. J. (1980) *J. Biol. Chem.* **255**, 1436–1441
- Sternweis, P. C., Northup, J. K., Smigel, M. D. and Gilman, A. G. (1981) *J. Biol. Chem.* **256**, 11517–11526
- Johnson, R. A. and Salomon, Y. (1991) *Methods Enzymol.* **195**, 3–21
- Brooker, G. (1988) *Methods Enzymol.* **159**, 45–50
- Steiner, A. L., Wehmann, R. E., Parker, C. W. and Kipnis, D. M. (1972) *Adv. Cyclic Nucl. Res.* **2**, 51–61
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Bradford, M. B. (1976) *Anal. Biochem.* **72**, 248–254
- Coleman, D. E., Berghuis, A. M., Lee, E., Linder, M. E., Gilman, A. G. and Sprang, S. E. (1994) *Science* **265**, 1405–1412
- Niroomand, F., Bangert, M., Phillips, C. and Rauch, B. (1993) *Mol. Pharmacol.* **43**, 90–95
- Seamon, K. B. and Daly, J. W. (1986) *Adv. Cyclic Nucl. Protein Phosphoryl. Res.* **20**, 1–150
- Alousi, A. A., Jasper, J. R., Insel, P. A. and Motulsky, H. J. (1991) *FASEB J.* **5**, 2300–2303
- Darfler, F. J., Mahan, L. C., Koachman, A. M. and Insel, P. A. (1982) *J. Biol. Chem.* **257**, 11901–11907
- Chidiac, P., Hebert, J. E., Valiquette, M., Dennis, M. and Bouvier, M. (1994) *Mol. Pharmacol.* **45**, 490–499
- Fraser, C. M. (1991) *Blood Vess.* **28**, 93–103
- Ostrowski, J., Kjelsberg, M. A., Caron, M. G. and Lefkowitz, R. J. (1992) *Annu. Rev. Pharmacol. Toxicol.* **32**, 167–183
- Noel, J. P., Hamm, H. E. and Sigler, P. B. (1993) *Nature (London)* **366**, 654–662
- Lambright, D. G., Noel, J. P. and Hamm, H. E. (1994) *Nature (London)* **369**, 621–628
- Sondek, J., Lambright, D. G., Noel, J. P., Hamm, H. E. and Sigler, P. B. (1994) *Nature (London)* **372**, 276–279
- Higashijima, T., Ferguson, K. M. and Sternweis, P. C. (1987) *J. Biol. Chem.* **262**, 3597–3602
- Higashijima, T., Ferguson, K. M., Sternweis, P. C., Smigel, M. D. and Gilman, A. G. (1987) *J. Biol. Chem.* **262**, 762–766
- Linder, M. E., Pang, I., Duronio, R. J., Gordon, J. I., Sternweis, P. C. and Gilman, A. G. (1991) *J. Biol. Chem.* **266**, 4654–4659
- Northup, J. K., Sternweis, P. C. and Gilman, A. G. (1983) *J. Biol. Chem.* **258**, 11361–11368
- Cerione, R. A., Staniszewski, C., Benovic, J. L., Lefkowitz, R. J., Caron, M. G., Gierschik, P., Somers, R., Spiegel, A. M., Codina, J. and Birnbaumer, L. (1985) *J. Biol. Chem.* **260**, 1493–1500
- Florio, V. A. and Sternweis, P. C. (1989) *J. Biol. Chem.* **264**, 3909–3915
- Brandt, D. R. and Ross, E. M. (1985) *J. Biol. Chem.* **261**, 1656–1664
- Lambright, D. G., Sondek, J., Böhm, A., Skiba, N. P., Hamm, H. E. and Sigler, P. B. (1996) *Nature (London)* **379**, 311–319
- Northup, J. K., Smigel, M. D., Sternweis, P. C. and Gilman, A. G. (1983) *J. Biol. Chem.* **258**, 11369–11376
- Gilman, A. G. (1992) *Cell* **70**, 869–872
- Watkins, D. C., Northup, J. K. and Malbon, C. C. (1987) *J. Biol. Chem.* **262**, 10651–10657
- Neubig, R. R. and Sklar, L. A. (1993) *Mol. Pharmacol.* **43**, 734–740
- Posner, R. G., Fay, S. P., Domalewski, M. D. and Sklar, L. A. (1994) *Mol. Pharmacol.* **45**, 65–73