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Quasi-irreversible binding of agonist to β-adrenoceptors and formation of non-dissociating receptor–G_s complex in the absence of guanine nucleotides

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Abstract

Here, we tested the hypothesis that receptor–G protein and agonist may form an irreversible complex in the absence of guanine nucleotides. We used the β-adrenoceptor–G_s system of guinea pig lung parenchymal membranes as a model. Two groups of membranes were used in the experiments: (1) washed with nucleotide-free buffer in the presence of isoproterenol (isoproterenol-treated), and (2) washed with buffer alone or with agonist + GDP (both were treated as control). Results were as follows: (1) the iodopindolol binding capacity of isoproterenol-treated membranes was reduced by about 30%. (2) No such reduction was observed in control membranes. (3) Addition of GDP to the isoproterenol-treated membranes completely restored the pindolol binding capacity. We interpreted this result as indicating irreversible agonist–receptor complex is formed when the receptor interacts with nucleotide-free $G_{s\alpha}$. (4) We observed a single peak of β_2-adrenoceptor activity in the control group by size-exclusion chromatography of the solubilized membranes. Inclusion of isoproterenol in the washing buffer led to an additional (heavier) peak of β_2-adrenoceptor activity. This peak disappeared when GDP was added to the detergent extract before high-pressure liquid chromatography (HPLC) analysis. Western blot analysis of these HPLC fractions showed that the agonist-induced heavier peak contained significantly more $G_{s\alpha}$ protein than did the other fractions. We interpreted this result as indicating that a practically irreversible complex of receptor and G protein is formed in the absence of GDP. We suggest that the tightly bound (nucleotide-free) receptor–G protein complex also contains the agonist, and that this complex can be reversed only by the addition of nucleotides. The implications of these results are also discussed.

Keywords: β-Adrenoceptor; G protein; Ternary complex; Irreversible complex; G protein–receptor interaction

1. Introduction

G proteins and associated receptors constitute a large family of signal transduction molecules that mediate transmembrane signaling of a number of hormones and neurotransmitters. The mechanism of G-protein-mediated signal transduction can be stated roughly in three steps: (1) The receptor is activated upon binding of ligand to the receptor, (2) the activated receptor activates the G protein (by "inducing" the exchange of bound GDP with GTP on the G protein), and finally (3) the activated G protein either interacts with an effector molecule to modulate its activity or it gets inactivated by its intrinsic GTPase activity. A great deal is known about the molecular details of the activation–inactivation cycle of G proteins as a result of the intensive studies carried out in the last decade (Noel et al., 1993; Lambright et al., 1994; Sprang, 1997). However, the exact mechanism of activation of G proteins by the receptor molecule is relatively obscure. One question about receptor-induced G protein activation concerns the mode of encounter of receptor and G protein in their native environment: are they physically precoupled or can they freely (and independently) diffuse in the plasma membrane (Stickle and Barber, 1993; Kenakin, 1996; Ugur and Onaran, 1997)? In the first case, the transmission of the signal that activates the receptor can be attributed only to the ability of receptor and G protein to adopt new conformations upon binding of the ligand to the receptor, which, in turn, determines the efficacy of the signal. In the second case, however, the probability of encounters and the duration of these encounters between receptor and G protein,

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which may well be modified by ligands, are additional parameters that can contribute to the process of receptor-mediated G protein activation. Although the literature on the topic is somewhat controversial (see Thomsen and Neubig, 1989; Neubig, 1994 for review), recent studies using experimental systems in which receptor and G protein are artificially fused, showed that the behavior of the fused proteins is indistinguishable in many respects from that of their native counterparts (Milligan and Rees, 1999; Milligan, 2000). This result can be taken as evidence for a lack of a significant contribution of the mode of encounter between receptor and G protein to the macroscopic properties of this signal transduction machinery in the cell membrane.

The activation of transducin (G_t) by photoreceptor rhodopsin is believed to occur in a different manner, in the sense that the activation of transducin by rhodopsin significantly depends on their proper collision in the membrane. Accordingly, an activated rhodopsin molecule can activate more than one molecule of G protein due to the multiple collisions that can occur during the free diffusion of the protein species, which obviously increases the efficiency of signal transduction. A mechanism that should further increase the efficiency of activation of a G protein in an encounter with a receptor has been suggested to be the formation of an irreversible complex between receptor and G protein, once GDP has dissociated from the G protein molecule. In this way, the contact between receptor and G protein never breaks until GTP (or GDP) binds to the G protein (Bruckert et al., 1992; Rodbell, 1997). Such a mechanism has not been established for other G protein systems.

The formation of an irreversible ternary complex in the absence of bound nucleotides may have implications for the interpretation of experimental data obtained with G protein systems other than the transducin system, and for the definition of fundamental parameters of receptor-mediated signal transduction such as ligand efficacy. For instance, if such an irreversible complex of ligand–receptor–(nucleotide-free)–G protein occurs, then the reversible ternary complex model (De Lean et al., 1980; Costa et al., 1992), which is almost exclusively used to explain nucleotide-sensitive and efficacy-dependent binding affinity of ligands, among many other phenomena, may not be an appropriate choice for defining and understanding ligand efficacy in molecular terms. This is because ligand efficacy becomes a kinetic (rather than equilibrium) property of the signal transduction system, and its measurement and interpretation become highly sensitive to the experimental conditions used to measure it. We therefore tested the irreversible ternary complex hypothesis for the β-adrenoceptor–G_s system in guinea pig lung parenchymal membranes that naturally express the β_2-adrenoceptor (80%), in which the GTP-induced shift in agonist affinity is also observable, and discussed the theoretical implications of the results.

2. Materials and methods

2.1. Preparation of crude membranes and treatment of the membranes

Crude lung parenchymal membranes were prepared from guinea pig lungs by homogenizing the tissue in ice-cold lysis buffer (5 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.2 mM phenylmethylsulphonylfloride, aprotinin 5 μg/ml). The homogenate was centrifuged at $500 \times g$ for 15 min, and the membrane pellets were obtained following centrifugation ($45\,000 \times g$ for 30 min, Sorval RC2-B) of the supernatant. Membranes were washed twice with buffer (50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA, 2 mM dithiothreitol, 0.2 mM phenylmethylsulphonylfloride, aprotinin 5 μg/ml) and stored (2–3 mg/ml of protein) in the same buffer including 25% sucrose at −70 °C. Before the experiments, membranes were thawed, divided into three treatment groups, and washed (1) in the presence of isoproterenol (100 μM) and in the absence of GDP. This was meant to increase the dissociation of GDP from G protein by agonist stimulation and to remove the released GDP from the solution, (2) in the presence of both isoproterenol and GDP (10 μM), and (3) in the absence of isoproterenol and GDP. The treatments were carried out in a Tris buffer (50 mM Tris and 100 mM KCl, pH 7.4) at a membrane concentration of 1.5 mg protein/ml. Briefly, membranes were suspended in the buffer and corresponding constituents for each group were added. After an incubation period of 15 min at 37 °C, membrane suspensions were spun at $45\,000 \times g$ (30 min) and resuspended in the Tris buffer. This procedure was repeated once more for each treatment group, and then membranes from each treatment group were washed three times by centrifuging and resuspending the pellets as above, using Tris buffer alone (without isoproterenol and GDP). The latter three washes eliminated additional components, namely isoproterenol and GDP, in the treatment groups.

2.2. Binding experiments

Binding of [^{125}I]iodopindolol (synthesized by using the chloramine T method (Barovsky and Brooker, 1980; Wolfe and Harden, 1981) and purified on reverse-phase high-pressure liquid chromatography (HPLC) to a specific activity of 2000 Ci/mmol) was measured in membranes (or in their detergent extracts) from the treatment groups. Competition binding of [^{125}I]iodopindolol with isoproterenol in the presence or absence of GppNHp was measured in triplicate after equilibration of the binding reaction (40 μg membrane protein) in binding buffer for 60 min at 37 °C (50 mM Tris at pH 7.4 and 100 mM KCl) in a total volume of 100 μl. Reactions were terminated by adding 3 ml of ice-cold binding buffer and rapid filtration through Whatman GF/B filters, using a vacuum filtration manifold (Milipore 1225). Filters were washed twice with 4 ml of

ice-cold binding buffer and counted for radioactivity. Time-dependent binding of a high concentration of [^{125}I]iodopindolol (200–400 pM) was determined by filtering 100-μl (20–40 μg of membrane) samples from a binding mixture, as described above, at indicated time points. In case of detergent extracts, we used ion exchanger filters (Whatman DE) to stop the reaction. The radioactivity on the filters was considered as total binding. Nonspecific binding was assessed by adding 200 μM propranolol to the reaction mixture in parallel experiments.

2.3. Solubilization and fractionation of receptors

The membrane preparations, treated as above (with isoproterenol, isoproterenol + GDP or buffer alone), were solubilized using *n*-octylglycoside or digitonin by gentle stirring on ice for 30–45 min in a solubilization buffer containing 1.2–1.5% of either detergent, 50 mM Tris, 1 mM EDTA, 100 mM KCl with a protein concentration of 3–5 mg/ml. Resulting suspensions were centrifuged at $40\,000 \times g$ for 1 h and supernatants were used for size-exclusion chromatography on a HPLC system (Jasco 880); 15–20% of specific [^{125}I]iodopindolol binding sites on the initial membrane preparations were recovered in the detergent extracts thus prepared. Hundred microliters of extracts from each treatment group was subjected to size exclusion chromatography using a column with a fractionation range of 10–400 kDa for globular proteins (Bio-Sil SEC250-5, 300 × 7.8 mm, Biorad). The column was eluted with the same solubilization buffer as the one used for membrane extraction, with a constant flow at 1 ml/min. The whole elution volume of the column (from 4.5 ml void to 11 ml total) was collected in 250-μl fractions. [^{125}I]iodopindolol binding sites and/or Gαs immunoreactivity were determined in the fractions.

2.4. Immunoblotting

HPLC fractions and plasma membranes were subjected to 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred electrophoretically to nitrocellulose membranes (Laemmli, 1970). Immunoblotting was performed using antiserum RM/1 ($G_{s\alpha}$) (NEN, USA) (dilutions 1:1000) and enhanced chemoluminescence (ECL). Briefly, nitrocellulose membranes were incubated overnight at 4 °C in phosphate-buffered saline (PBS) containing 3% bovine serum albumin and 8% nonfat dry milk. Blots were washed several times with PBS, then incubated with antiserum at room temperature for 1–2 h. Blots were then washed several times with PBS and incubated with horseradish peroxidase-labelled donkey anti-rabbit IgG (Amersham, UK) for 1 h at room temperature. Blots were washed several times with PBS and incubated with ECL western blotting reagent (Amersham) for 1 min and exposed to X-ray film for 15–45 s.

2.5. Chemicals and other procedures

Protein concentration of the preparations was determined according to the method of Bradford (1976). Mean values were compared using Student's statistics. Probability values less than 0.05 were considered significant. Competition binding curves were evaluated by fitting to a four-parameter-logistic equation. (−)-Pindolol was purchased from RBI, and guanine nucleotides from Boehringer Manheim. ^{125}I–NaI was from Amersham. All other chemicals were from Sigma at highest possible purity.

3. Results

3.1. Reduction of iodopindolol binding sites in agonist-treated membranes under nucleotide-free conditions

As described in the experimental procedures, the membranes were washed under three different conditions: (i) in the presence of agonist (isoproterenol) and in the absence of GDP, (ii) in the presence of both agonist and nucleotide, and (iii) in the absence of agonist and nucleotide, in which iodopindolol binding capacity was assessed. The first group will be referred to as "isoproterenol-washed" and the last two groups as "controls".

No difference was observed in iodopindolol binding capacity within the control groups. However, the iodopindolol binding capacity of the isoproterenol-washed membranes was reduced significantly when compared to that of the control membranes (~ 30% reduction on average). Fig. 1 shows representative data from an experiment. The

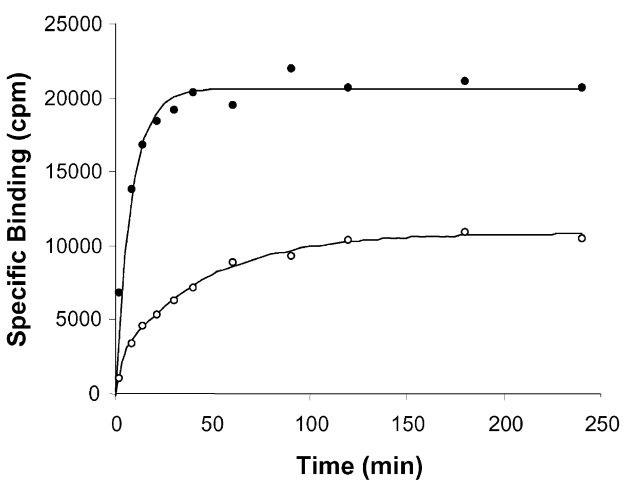

Fig. 1. Time-dependent specific binding of ^{125}I-iodopindolol to isoproterenol-washed guinea pig lung membranes. Membranes were washed in the presence of isoproterenol and in the absence (open symbols) or presence (closed symbols) of GDP as described in methods. ^{125}I-iodopindolol (500 kcpm/ml) was added at time 0, and specific binding was measured at indicated time points in 100-μl samples, which were drawn from the incubation mixtures. Nonspecific binding was determined in a parallel experiment in the presence of (−)-propranolol (200 μM). Membrane concentration was 25 μg/100 μl. Solid curves are the best fits of simple exponential growth.

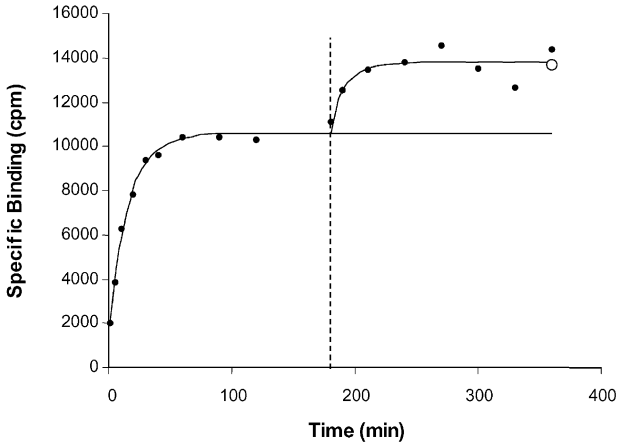

Fig. 2. Time-dependent specific binding of ^{125}I-iodopindolol to isoproterenol-washed guinea pig lung membranes. Membranes were washed in the presence of isoproterenol and in the absence of guanine nucleotides as described in methods. ^{125}I-iodopidolol (500 kcpm/ml) was added at time 0 and specific binding was measured at the indicated time points in 100-μl samples, which were drawn from the incubation mixture. Nonspecific binding was determined in a parallel experiment in the presence of (−)-propranolol (200 μM). GDP was added (100 μM) to the incubation mixture at the indicated time (dotted vertical line). Membrane concentration was 20 μg/100 μl. Solid curves are the best fits of simple exponential growth, starting from time 0 or from the time point where GDP was added. The open circle shown at the last time point indicates the specific binding measured in a parallel experiment in which the membranes were washed in the absence of isoproterenol but according to the same protocol as described above.

apparent reduction of iodopindolol binding sites in these membranes cannot be explained by (i) the expected effect of a residual agonist concentration in the solution after washout, which may compete with the radioligand binding, or (ii) by an agonist-induced "desensitization" of the receptors during such a long incubation period, for the following reasons. First, one of the control groups (the second one mentioned above) also contained isoproterenol and GDP, and no such reduction in iodopindolol binding sites was observed. Second, we directly measured isoproterenol concentration in the solution during the washout protocol described in Materials and methods section by using an electrochemical detector and a high-pressure liquid chromatography system, and we found that the residual isoproterenol concentration was lower than 10 nM ($\sim 1/20$ of its K_d) at the end of the washout protocol. Third, addition of GDP to the isoproterenol-washed membranes in the middle of the binding experiment immediately restored the iodopindolol binding capacity of the membranes to the control level. Representative data from such an experiment are shown in Fig. 2. The first two points show that it was not the agonist contamination in the binding buffers that reduced iodopindolol binding. The third point (along with the first one) excludes the possibility that the receptors underwent "desensitization" such as internalization, which is anyway unlikely to occur in broken cells. Thus, the apparent reduction in iodopindolol binding sites requires not only the presence of agonist but also the absence of GDP during the incubation period. The GDP-restorable reduction in iodopindolol binding sites was not surmountable by increasing the concentration of radioligand (data not shown). Thus, the possibility that the apparent increase in iodopindolol binding—observed at fixed concentrations of iodopindolol—is due to a nucleotide-induced increase in iodopindolol affinity (which may well be the case for negative antagonists) can be excluded. We, therefore, concluded that tight binding of isoproterenol to the receptors in the absence of GDP is responsible for the apparent reduction in binding sites in isoproterenol-washed membranes.

3.2. Formation of an irreversible receptor–G protein complex

Considering the GDP dependence of the agonist-induced reduction in β-adrenoceptor binding sites, we evaluated the possibility that receptor and G_s protein may also form a quasi-irreversible complex in the absence of GDP, to which agonist can bind irreversibly. Thus, after the washing procedure (described above), we solubilized the membranes by using digitonin or octylglucoside and subjected the preparation to size-exclusion chromatography. In the eluted fractions, we measured β-adrenoceptor binding activity and $G_{s\alpha}$ immunoreactivity by iodopindolol binding and SDS-PAGE-immunoblotting, respectively. A representative experiment with digitonin is shown in Fig. 3. The

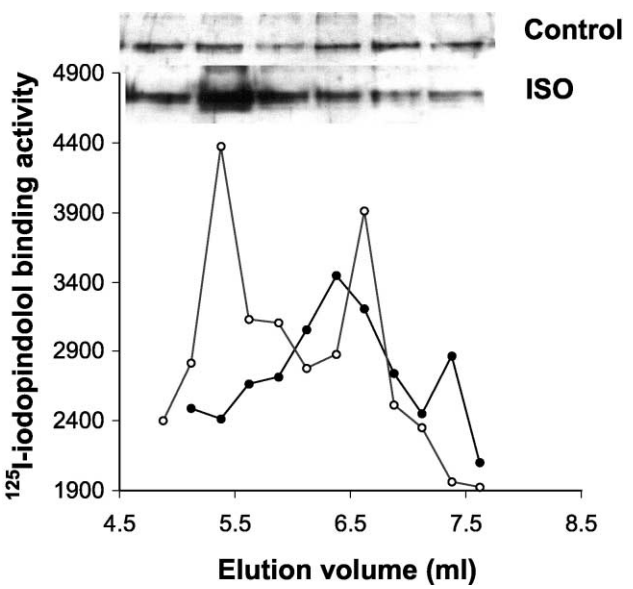

Fig. 3. Size-exclusion HPLC chromatogram of digitonin-solubulized guinea pig lung membranes. ^{125}I-iodopidolol binding activities of fractions are shown on the ordinate and $G_{s\alpha}$ immunoreactivity of the corresponding fractions in Western blots are shown in the upper part of the figure. Two types of membranes were used: (1) washed in the absence of isoproterenol and GDP (closed symbols in the chromatogram and indicated as control in the blot) and (2) washed in the presence of isoproterenol but in the absence of GDP (open symbols in the chromatogram and indicated as isoproterenol in the blot). See Materials and methods for the washing protocols and other technical details.

control membranes showed a single peak of β-adrenoceptor binding activity, whereas the isoproterenol-washed membranes (in the absence of GDP) possessed an additional (heavier) peak of β-adrenoceptor binding activity. The latter peak coincided with an intensified G_s immunoreactivity (compared to the control), suggesting an agonist-induced formation of a stable complex of β-adrenoceptor–G_s in the absence of GDP. We obtained very similar results with octylglycoside (data not shown).

3.3. Dependence of GTP shift in agonist affinity on the concentration of membrane

The above result suggests that the high-affinity agonist binding observed in binding experiments in the absence of guanine nucleotides (and thus the extent of nucleotide-induced affinity shift) may be due to the formation of a quasi-irreversible complex of ligand–receptor and nucleotide-free G protein. This scenario is actually different from what is implied in the usual ternary complex model. In the ternary complex model, the affinity of receptor and G protein binding is modified by an agonist by a factor of α (or vice versa), which is assumed to be the origin of the nucleotide-induced shift in agonist apparent affinity and to be the result of strict-sense reversible interactions. In the above scenario, however, the nucleotide shift in binding may result from the formation of an irreversible complex, the affinity of which is expected to be infinite in the absolute absence of guanine nucleotides (see discussion for a more detailed explanation of the scenario). If this is the case, dilution of the contaminating GDP, which may come from the membrane preparations, should have some effect on high-affinity agonist binding in the binding experiments. We tested this hypothesis by performing agonist competition binding experiments in a total volume of 100 or 1000 μl. We kept the concentration of radioligand and the amount of membrane constant in these experiments. Fig. 4 shows representative results from these experiments. The guanine nucleotide-induced shift in apparent affinity was greater when the experiment was performed in 1000 μl, as predicted from the above scenario (see discussion).

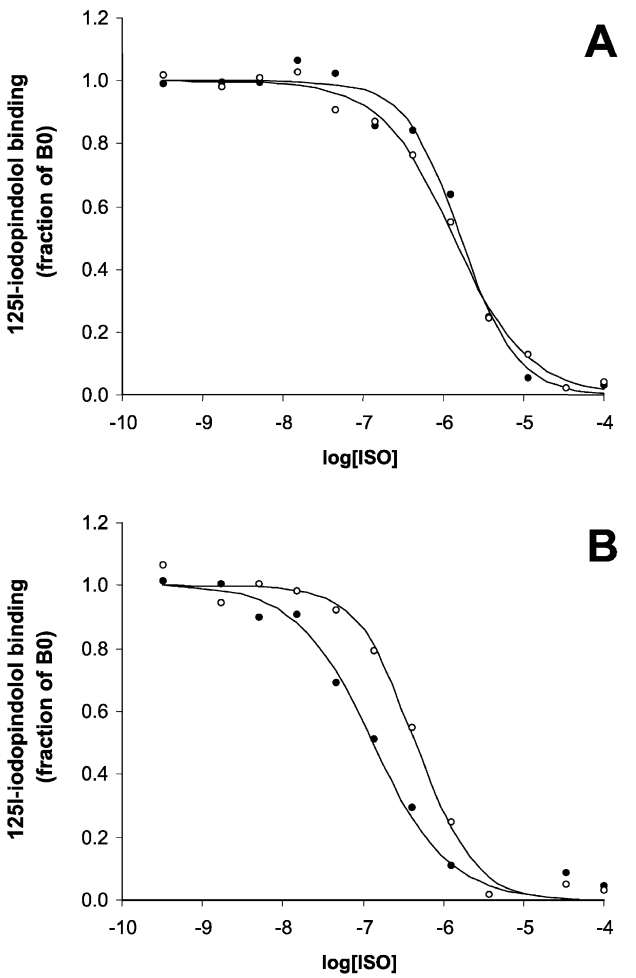

Fig. 4. Competition binding of isoproterenol (with ^{125}I-iodopidolol) in guinea pig lung membranes. Competition binding was measured in 100 (A) or 1000 (B) μl volumes in the absence (closed circles) or presence (open circles) of GDP 100 μM. ^{125}I-iodopidolol concentration was constant in A and B (20 pM), but the membrane concentration was 10 times higher in A than in B (30 μg/tube in both experiments but volumes of incubation was different). Results are mean values of triplicate determinations. Solid curves represent best fit of four-parameter logistic equation. Data were normalized with respect to fitted B_0 in corresponding curves.

4. Discussion

In this study, we investigated the possibility of the formation of an irreversible complex of agonist, receptor and guanine nucleotide-free G protein in the β_2-adrenoceptor–G_s system. Such a mechanism, which may increase the catalytic efficiency of activation of G protein by the activated receptor, has actually been suggested for the rhodopsin–transducin system (Bruckert et al., 1992), but has not been discussed explicitly for other G protein systems. Here, we provide still indirect but suggestive evidence for the formation of such a complex in the β-adrenoceptor–G_s system. In the following paragraphs, we briefly mention the literature that led us to this study and discuss the implications of an irreversible step in G protein activation.

It has long been considered that ligand, receptor and G protein form a high-affinity ternary complex in the absence of guanine nucleotides, and that the complex is transient in the presence of guanine nucleotides. However, whether the G protein in the "high-affinity" complex is in its inactive GDP-bound heterotrimeric form or in its nucleotide-free intermediate state has always been obscure. Close inspection of the literature in the field may provide some clues. Although these studies have been performed with totally different ideas in mind, when their experimental design and results are considered together, they suggest that ago-

nist, receptor and G protein form an almost irreversible complex (i.e. more than a high-affinity complex) in the absence of guanine nucleotides. These studies date back to the late seventies. For instance, by using size-exclusion chromatography, Limbird and Lefkowitz (1978) have shown that agonist increases the β-adrenoceptor "size" in solubilized membranes. In their experimental design, the agonist–receptor (and presumably G protein) complex survives several hours despite the extensive dilution of ligand (as well as guanine nucleotides). Likewise, solubilized complexes of α-adrenoceptors (Smith and Limbird, 1981), dopamine receptors (Kirpatrick and Caron, 1983), vasopressin receptors (Fitzgerald et al., 1986) or angiotensin receptors (De Lean et al., 1984) with their respective agonists survive minutes to several hours under diluting conditions for both ligand and guanine nucleotides. The stability of these complexes has always been found to be sensitive to added nucleotides (i.e. GDP or GTP), suggesting that the G protein should also be there in these complexes, except in a study where irreversible ligand–β-adrenoceptor complex formation has been claimed to be induced by detergent (deoxycholate) (Neufeld et al., 1983). The present results also show that the agonist-induced ternary complex in solution is practically irreversible when care is taken to remove GDP during the incubation of membranes with agonist before solubilization and that the complex dissociates when GDP is added before or after solubilization.

Such an irreversible step, in which a receptor–(nucleotide-free) G protein complex is involved, has been explicitly formulated for the rhodopsin–transducin system (Bruckert et al., 1992). Transducin has been shown to bind nucleotides irreversibly in the absence of bound (active) rhodopsin, and rhodopsin has been shown to bind transducin irreversibly in the absence of bound nucleotides. Such a model (including the ligand–receptor interaction) is illustrated in Fig. 5. One mechanistic implication of this model is that once G protein is caught by the active receptor, then the GDP dissociates and the nucleotide-free form of the G protein stays stable until GTP (or less probably GDP) binds. The catalytic efficiency of the receptor to activate G protein should be higher in this case than

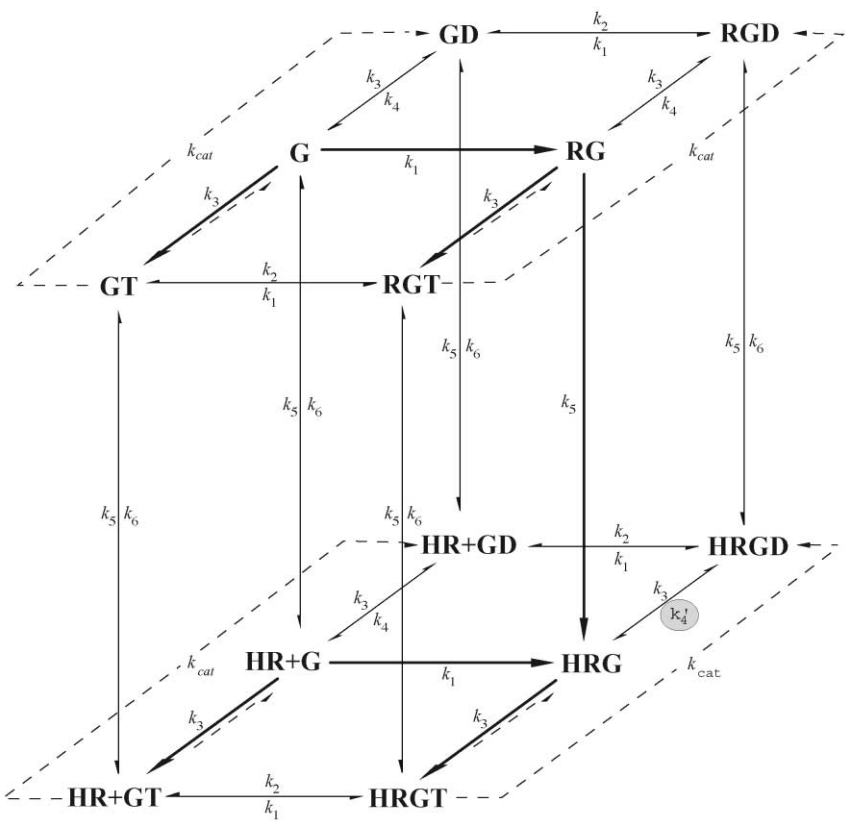

Fig. 5. The reaction scheme inferred from the interaction model proposed by Bruckert et al. (1992) for transducin. H, R, G, D and T signify hormone, receptor, G protein (α subunit), GDP and GTP, respectively. Note that GTP binding to any form of G, receptor binding to empty G and hormone binding to receptor-empty G complex are all assumed to be irreversible. Rate constants are indicated next to the corresponding reaction arrows. Transition from upper surface to the lower one indicates hormone binding to the relevant species (with rate constants $[k_5,k_6]$ independent of the identity of the complex that include receptor). Likewise, transition from the left side to the right side represents the binding of receptor to G protein in all possible ways (with constant rate constants $[k_1,k_2]$). The association rate of GDP or GTP to G protein is assumed to be constant and identical for both nucleotides (k_3); and only the dissociation rate of GDP is assumed to be dependent on whether the interacting receptor is hormone bound or not (k_4' or k_4, respectively). The only "state-sensitive" rate constant, namely k_4', is marked in the scheme. k_{cat} indicates the catalytic rate of GTPase reaction.

when nucleotide-free G protein and active receptor can dissociate (see Bruckert et al., 1992 for discussion). This mechanism may indeed serve to increase the activation efficiency of the G protein by the agonist-bound receptor under physiological conditions, where nucleotide concentrations are very high.

Another interesting implication of this model for those receptors that are activated by reversibly binding ligands is that the guanine nucleotide-induced shift in agonist binding affinity observed in binding experiments seems to be a kinetic rather than an equilibrium phenomenon. In the ternary complex formalism, the origin of the GTP-induced shift in agonist binding has been attributed to the difference between the affinity of ligand for "naked" receptor and G protein-bound receptor (which has been assumed to be uncoupled by GTP). The difference in these affinities has been expressed as an allosteric coupling factor α in the ternary complex model (Costa et al., 1992); α has been thought to reflect the molecular efficacy of the ligand at the receptor, which can be theoretically measured as the difference in the binding affinity of the ligand at the receptor in the absence and in the presence of a saturating amount of G protein. Thus, the ternary complex (and related) models see α as a constant for a particular triplet of ligand, receptor and G protein. However, according to the above scenario, α becomes a function of contaminating [GDP] in the membrane, in which case it can no longer be used as a (constant) measure of ligand efficacy. In order to show this behavior more concretely, we simulated an agonist binding experiment by evaluating the irreversible-step model at steady state. Fig. 6 shows the results of a simulation in which we calculated the steady-state binding of an agonist in the presence of different concentrations of GDP. It is evident from this simulation that (1) GDP concentration (as low as the G protein concentration) sets the high-affinity value of agonist binding, (2) increasing the GDP concentration shifts the agonist binding toward the ultimate low-affinity binding, and (3) as the concentration of GDP decreases below the G protein concentration, an irreversible part of agonist binding becomes apparent. Thus, if we assume that the agonist affinity observed with the high concentration of GDP is the low-affinity binding of agonist, then the initial value of contaminating GDP (that may come from the membrane) determines the magnitude of the guanine nucleotide-induced shift in agonist affinity. This prediction is consistent with the experimental results given in Fig. 4. In this experiment, (competition) binding of isoproterenol was evaluated in the presence and absence of added GDP in two different volumes (ligand concentrations were kept constant but the membrane concentration was different in two different volumes). As one would expect from the above scenario, the GTP-induced shift was larger in the high-volume than in the low-volume experiment since membrane-bound GDP would be more diluted in the latter case than in the former. Note that in this simulation agonist was assumed to be modifying only

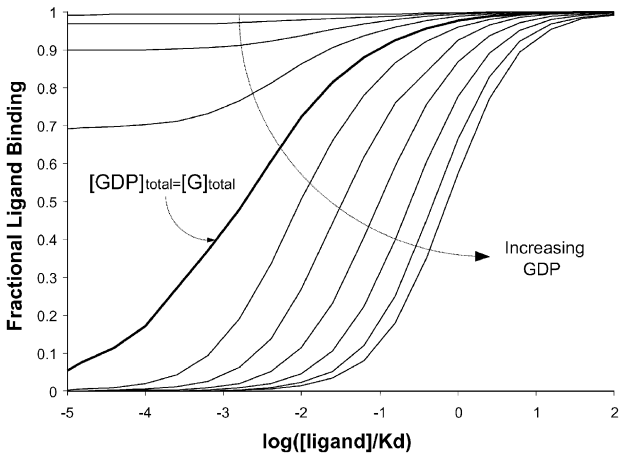

Fig. 6. Computer simulation of steady-state agonist binding to receptor in the absence of GTP and in the presence of both G protein and GDP, using the model shown in Fig. 5. GDP concentration is variable as indicated in the figure. Agonist concentration was normalized with respect to its (unconditional) equilibrium dissociation constant ($K_d = k_6 / k_5$). Ligand binding is given as the fraction of total receptor concentration. Receptor and G protein concentrations were assumed to be equal (100 pM). GDP concentration changed from 1 pM (upper most curve) to 100 nM (right most curve) in equally spaced logarithmic steps. Agonist concentration was assumed to be unaffected by its binding to the receptor. Rate constants were as follows: agonist association (k_5) = 1 and dissociation (k_6) = 0.1, receptor–G protein association (k_1) = 10 000 and dissociation (k_2) = 1, GDP association (k_3) = 100 and dissociation (k_4) = 10^{-8} (in the absence of bound agonist–receptor complex), (k'_4) = 10 (in the presence of bound agonist–receptor complex). Association and dissociation constants have the unit of $\mu M^{-1}\ s^{-1}$ and s^{-1}, respectively. Absolute values of rate constants were chosen arbitrarily because they do not have any effect on the general conclusion we want to draw from the simulation. However, the association constant of receptor–G protein binding was relatively high considering the relatively high planar diffusional accessibility of the two species in the membrane. Agonist was simulated as inducing the dissociation of GDP from G protein (compare k'_4 and k_4). Steady-state solutions were obtained using the freely distributed software GEPASI v.2.08, Pedro Mendes 1992/93. Note that the total GDP concentration (as low as total G protein concentration) determined the apparent (high) affinity of agonist binding, and that when the total GDP concentration was lower than that of G protein, then irreversible binding of agonist became apparent.

the dissociation rate of GDP from the G protein; all other rate constants were independent of the ligation states of the interacting partners, namely receptor, G protein, nucleotide and agonist. This implies that the ligand efficacy can be considered as the ability of the ligand to modify the dissociation rate of GDP from G protein, by changing the state of the receptor upon ligation. However, to the best of our knowledge, there are no available experimental data that show systematically for a set of ligands the correlation between the ability of ligand to modify effector activation (i.e. observed efficacy) and its ability to modify the dissociation rate constant of GDP from G protein.

Finally, a molecular model has recently been reported that can be used to analyze the interaction of receptor and G proteins (by using molecular modeling, rigid body docking and molecular dynamics techniques) (Fanelli et al.,

1999). It would be an interesting challenge to build an empty G protein model to see whether the method can predict relatively strong interactions between this form of G protein and the receptor.

In conclusion, nucleotide-free $G_{\alpha s}$ forms an apparently irreversible complex with the β_2-adrenoceptor, which, in turn, binds the agonist isoproterenol irreversibly in this state. Such a state can be induced experimentally by removing the dissociating nucleotide from the G protein. This can be considered as a mechanism that maximizes the efficiency of agonist-mediated G protein activation in the presence of both GDP and GTP. Such a mechanism calls for a reconsideration of the concept that molecular ligand efficacy can be identified by means of their free energy coupling factors in the context of the ternary complex model.

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