High affinity agonist binding to G protein-coupled receptors depends on the formation of a ternary complex between agonist, receptor, and G protein. This process is too slow to be accounted for by a simple diffusion-controlled mechanism. We have tested if the interaction between activated receptor and G protein is rate-limiting by fusing the coding sequence of the human A1-adenosine receptor to that of Gα1 (A/Gα1) and of Gαo (A/Gαo). Fusion proteins of the expected molecular mass were detected following transfection of HEK293 cells. Ternary complex formation was monitored by determining the kinetics for binding of the high affinity agonist (−)-N6-3[125I](iodo-4-hydroxyphenylisopropyl)adenosine; these were similar in the wild-type receptor and the fusion proteins over the temperature range of 10 to 30 °C. Agonist dissociation may be limited by the stability of the ternary complex. This assumption was tested by creating fusion proteins in which the Cys351 of Gα1 was replaced with glycine (A1/Gα1C351I) to lower the affinity of the receptor for the G protein. In these mutated fusion proteins, the dissociation rate of the ternary complex was accelerated; in contrast, the rate of the forward reaction was not affected. We therefore conclude that (i) receptor activation per se rather than its interaction with the G protein is rate-limiting in ternary complex formation; (ii) the stability of the ternary complex is determined by the dissociation rate of the G protein. These features provide for a kinetic proofreading mechanism that sustains the fidelity of receptor-G protein coupling.

High affinity agonist binding to G protein-coupled receptors depends on the formation of a ternary complex between agonist, receptor, and G protein. This process is too slow to be accounted for by a simple diffusion-controlled mechanism. We have tested if the interaction between activated receptor and G protein is rate-limiting by fusing the coding sequence of the human A1-adenosine receptor to that of Gα1 (A/Gα1) and of Gαo (A/Gαo). Fusion proteins of the expected molecular mass were detected following transfection of HEK293 cells. Ternary complex formation was monitored by determining the kinetics for binding of the high affinity agonist (−)-N6-3[125I](iodo-4-hydroxyphenylisopropyl)adenosine; these were similar in the wild-type receptor and the fusion proteins over the temperature range of 10 to 30 °C. Agonist dissociation may be limited by the stability of the ternary complex. This assumption was tested by creating fusion proteins in which the Cys351 of Gα1 was replaced with glycine (A1/Gα1C351I) to lower the affinity of the receptor for the G protein. In these mutated fusion proteins, the dissociation rate of the ternary complex was accelerated; in contrast, the rate of the forward reaction was not affected. We therefore conclude that (i) receptor activation per se rather than its interaction with the G protein is rate-limiting in ternary complex formation; (ii) the stability of the ternary complex is determined by the dissociation rate of the G protein. These features provide for a kinetic proofreading mechanism that sustains the fidelity of receptor-G protein coupling.

Whether the conformational transition to R* is rate-limiting or whether ternary complex formation is limited by the association of receptor and G protein (2, 3). Based on kinetic arguments, it has been suggested that a significant portion of the receptors were precoupled, i.e. there is a fraction of preformed R/G complexes in intact cells and membranes because receptors can bind G proteins in the absence of agonists (for review, see Refs. 4–6). However, earlier experiments that were designed to investigate the kinetics of adenylyl cyclase regulation by the α2-adrenergic receptor failed to detect an initial burst of receptor-dependent inhibition although this would be predicted for precoupled receptors (7). Similarly, the mechanism of signal transduction in the visual system is inconsistent with precoupling of rhodopsin and transducin (8).

In contrast to photoreceptors (or other specialized sensory cells) where a receptor is only confronted with a single type of G protein, in most cells a given receptor selects its cognate G protein(s) from a multitude of available G protein oligomers; the specificity in this interaction that can be observed is remarkable in many instances (reviewed in Ref. 9). The mechanism by which this fidelity is achieved is not fully understood. In the present work, we used the human A1-adenosine receptor because its interaction with G proteins has been extensively characterized in experiments with purified and defined components (10–15); we have generated precoupled R/G tandems by fusing the coding sequence of the human A1-adenosine receptor to that of Gα1 (or Gαo) to test if the formation of the ternary complex was limited by the association of receptor and G protein. In addition, we have altered the affinity of the G protein for the receptor by introducing mutations at the carboxyl terminus of the Gα subunit, a site that is critical for R/G interaction (16, 17). Using this approach, we show that the association of receptor and G protein is not rate-limiting; in contrast, the stability of the ternary complex is limited by the dissociation rate of the G protein. This suggests that the fidelity of receptor-G protein coupling is achieved by a kinetic proofreading mechanism.

### EXPERIMENTAL PROCEDURES

**Materials**—Adenosine deaminase and Complete™ protease inhibitor tablets were from Roche Molecular Biochemicals (Germany). Heps and CHAPS were from BIOSID (Munich, Federal Republic of Germany); suramin was obtained from Research Biochemicals (Natick, MA). The materials required for SDS-polyacrylamide gel electrophoresis were from BioRad. Fetal calf serum was from PAA Laboratories (Linz, Austria). Dulbecco’s modified Eagle’s medium, non-essential amino acids, β-mercaptoethanol, 1-glutamine, penicillin G, streptomycin, and G418 (geneticin) were obtained from Life Technologies, Inc. (Grand Island, NY). CAMP, CPA, forskolin, and pertussis toxin were purchased from Sigma-Aldrich. Buffers and salts were from Merck (Darmstadt, Germany). [3H]adenine and [125I]HPIA were from NEN Life Science Products Inc. (Boston, MA). [125I]HPIA was synthesized according to Linden (18). Centrifuge tubes and tissue culture plates were from Greiner (Vienna, Austria) and Corning Costar (Acton, MA). Plasmid preparation kits were
Table I

| Ligand Binding to Fused A1-Adenosine Receptor/G Protein Tandems |
|---------------------------------------------------------------|
| **Association and dissociation rate constants for binding of [125I]HPIA to the A1-adenosine receptor and fusion proteins comprising the receptor and the indicated G protein α-subunits** |
| **k_{app}, k_{off}, k_{on}** | **min^{-1}, s^{-1}, M^{-1} s^{-1}** |
| **A1** | 0.043 ± 0.006 | 0.020 ± 0.004 | 4.77 × 10^{10} |
| **A1/Gi60** | 0.048 ± 0.002 | 0.018 ± 0.002 | 4.95 × 10^{10} |
| **A1/Gi60-i-1** | 0.057 ± 0.009 | 0.021 ± 0.004 | 5.97 × 10^{10} |
| **A1/Gi60-i-1C351G** | 0.124 ± 0.027 | 0.107 ± 0.027 | 4.49 × 10^{10} |

The apparent dissociation constant (k_{app}) and the dissociation rate (k_{off}) were derived from experimental data as outlined in the legends to Figs. 4 and 8; the incubation temperature was 25 °C. The experimental data points were fitted to monoequivalent equations or to equations describing the sum of two exponential processes; the latter models did not improve the fit significantly (p > 0.05 in all cases, F-test). The association rate (k_{on}) was calculated from the relation: k_{on} = (k_{app} - k_{off})/L, where L denotes the radioligand concentration (0.92–1.1 nM). Data are means ± S.E. of three to six experiments carried out in duplicate.
brane. The A1-adenosine receptor is coupled to the pertussis toxin-sensitive G proteins of the Gi/Go group (10–15). HEK293 cells stably transfected with the human A1-adenosine receptor were treated with pertussis toxin (100 ng/ml for 24 h) to fully inactivate the endogenous G proteins (see Fig. 6). Membranes prepared from these cells were incubated with 10 nM (●) or 50 nM (○) rGoα,1 and amounted to less than 10% of the total binding seen with 10 nM rGoα,1 which had been combined with a 4-fold molar excess of purified brain βγ-dimer in the presence of 10 nM CHAPS. The binding reaction was started by adding buffer prewarmed to 25 °C and containing [125I]HPIA (0.5 nM final concentration) yielding a final CHAPS concentration of 2.5 mM. Aliquots were withdrawn at the time points indicated and immediately filtered over glass fiber filters. Data are expressed as percent of the respective Bmax values which were 66 ± 9 fmol/mg for 10 nM and 146 ± 23 fmol/mg for 50 nM rGoα,1. Nonspecific binding determined in the presence of 1 μM CPA did not change over the time course of the assay and amounted to less than 10% of the total binding seen with 10 nM rGoα,1. koff was estimated to be 0.0243 ± 0.004 min⁻¹ for 10 nM and 0.0271 ± 0.003 min⁻¹ for 50 nM rGoα,1, respectively. Panel B, concentration-dependent increase in the apparent association rate constant k on of [125I]HPIA. The apparent association rate constant was determined at the indicated concentrations of [125I]HPIA; the binding reaction was carried out with membranes from HEK293 cells expressing the A1-adenosine receptor. Assay conditions were as described for panel A. The pseudo-first order rates were calculated by nonlinear least squares curve fitting and the linear regression of the secondary plot was calculated. The y intercept yielding an estimate of k on was 0.024 min⁻¹; k on estimated from the slope of the regression line was 4.2 × 10⁻⁶ min⁻¹ s⁻¹. The dotted lines indicate the 95% confidence interval.

The interaction between receptor and G protein is essentially confined to a two-dimensional plane, i.e. the inner leaflet of the lipid bilayer, that is limited by the size of the vesicle. Because G proteins cannot be inserted into an individual vesicle to arbitrarily high levels, the variation in the concentration of G proteins that can be achieved in the vicinity of the receptor is presumably modest. As an alternative, the diffusion step, in which the activated receptor collides with the appropriate G protein, can be eliminated by fusing the receptor directly to the G protein α-subunit.

Expression of the A1-adenosine Receptor/Gα Fusion Proteins—We have verified that fusion proteins consisting of the A1-adenosine receptor and of the Gα subunits were expressed by immunodetecting the A1rGoα,1 and the A1rGoα construct with appropriate antisera (Fig. 2A). Diffuse bands in the 70–80-kDa range were observed in the lanes which contained membrane proteins of cells transfected with the plasmids encoding the fusion proteins. These were absent in untransfected cells or in cells expressing the A1-adenosine receptor. The apparent molecular mass of the purified A1-adenosine receptor is ~35 kDa (28), while that of Goα,1 and of Goα are 39 and 41 kDa, respectively. The migration of molecular mass markers are indicated.

Fig. 1. Panel A, association of the agonist radioligand [125I]HPIA to the human A1-adenosine receptor reconstituted with 10 nM (●) or 50 nM (○) rGoα,1. Membranes (~10 μg of protein) prepared from pertussis toxin-treated cells expressing the human A1-adenosine receptor were preincubated with 10 nM (●) or 50 nM (○) rGoα,1, which had been combined with a 4-fold molar excess of purified brain βγ-dimer in the presence of 10 nM CHAPS. The binding reaction was started by adding buffer prewarmed to 25 °C and containing [125I]HPIA (0.5 nM final concentration) yielding a final CHAPS concentration of 2.5 mM. Aliquots were withdrawn at the time points indicated and immediately filtered over glass fiber filters. Data are expressed as percent of the respective Bmax values which were 66 ± 9 fmol/mg for 10 nM and 146 ± 23 fmol/mg for 50 nM rGoα,1. Nonspecific binding determined in the presence of 1 μM CPA did not change over the time course of the assay and amounted to less than 10% of the total binding seen with 10 nM rGoα,1. koff was estimated to be 0.0243 ± 0.004 min⁻¹ for 10 nM and 0.0271 ± 0.003 min⁻¹ for 50 nM rGoα,1, respectively. Panel B, concentration-dependent increase in the apparent association rate constant k on of [125I]HPIA. The apparent association rate constant was determined at the indicated concentrations of [125I]HPIA; the binding reaction was carried out with membranes from HEK293 cells expressing the A1-adenosine receptor. Assay conditions were as described for panel A. The pseudo-first order rates were calculated by nonlinear least squares curve fitting and the linear regression of the secondary plot was calculated. The y intercept yielding an estimate of k on was 0.024 min⁻¹; k on estimated from the slope of the regression line was 4.2 × 10⁻⁶ min⁻¹ s⁻¹. The dotted lines indicate the 95% confidence interval.

Fig. 2. Immunodetection of fusion proteins comprising the A1-adenosine receptor and Goα,1, or Goα stably expressed in HEK293 cells. Panel A, membrane protein (50 μg/lane) from untransfected control cells (lane 2), HEK293 cells expressing the A1-adenosine receptor (lane 3), the A1rGoα,1 (lane 4), or the A1rGoα fusion protein (lane 1) were resolved on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. The blot was probed sequentially with antiserum I1C (50 ng) was applied as a standard. Panel B, two aliquots (50 μg/lane) of HEK293 cell membranes containing the A1-adenosine receptor (lanes 2 and 4) or the A1rGoα fusion protein (lanes 1 and 3) were applied onto a 10% SDS-polyacrylamide gel. After electrophoretic transfer, the nitrocellulose membrane was cut into halves; these were probed with antibodies (i.e. antisera ON1 and OC2 which are directed against the amino and carboxyl terminus of Goα, respectively. The migration of molecular mass markers are indicated.
respectively. Thus, the size of the immunoreactive material is consistent with the expected molecular mass of the fusion proteins; the broad staining pattern presumably arises from microheterogeneity due to glycosylation of the receptor moiety in the fusion protein rather than from partial proteolysis. We have employed the antisera ON1 and OC2 which are directed against the amino and carboxyl terminus of Goα, respectively (26). Immunostaining with these two antisera visualized a predominant band in the range of 70–80 kDa; in addition, antiserum ON1 detected a band at about 50 kDa (lane 1, Fig. 2B). This band was not seen in cells lacking the fusion protein (lane 2, Fig. 2B) and in the immunoblot with antiserum OC2 (lane 3 in Fig. 2B). Because this band retains the amineterminal epitope, it may result from proteolysis within the Goα moiety of the fusion protein. It is, however, evident that this cleavage product represents only a very minor fraction of the total immunoreactivity. We therefore conclude that the bulk of the fusion proteins comprise an intact G protein moiety. Saturation binding experiments with the antagonist radioligand [3H]DPCPX revealed that the fusion proteins A1/Gαi,1 and A1/Gαo were stably expressed up to levels of ~5 and 10 pmol/mg, respectively. Because HEK293 cells do not express detectable levels of Goα, we subsequently present data on the comparison of the fusion protein A1/Gαi,1 with the wild-type receptor (which interacts with the Goα complement endogenous to HEK 293 cells). We stress, however, that the ligand binding kinetics of the fusion protein A1/Gαi were essentially identical to that of A1/Gαo (data not shown; see also Table I).

The association (Fig. 3A) and dissociation kinetics (Fig. 3B) of the antagonist [3H]DPCPX were found to be virtually identical in binding assays that were carried out in parallel with membranes carrying the unfused A1-adenosine receptor (○ in Fig. 3, A and B) and those containing the fusion protein A1/Gαi,1 (● in Fig. 3, A and B). For the A1-adenosine receptor, the apparent (pseudo-first order) association and dissociation rates were calculated as 0.23 ± 0.05 min⁻¹ and 0.14 ± 0.06 min⁻¹, respectively; the corresponding values for the fusion protein A1/Gαi,1 were kapp = 0.37 ± 0.08 min⁻¹ and kad = 0.16 ± 0.05 min⁻¹. The kinetically derived estimates for the dissociation constant KD were 1.5 and 0.76 nM for the A1-adenosine receptor and for A1/Gαi,1, respectively. The capacity of the A1/Gαi,1 tandem to impinge on an effector was investigated by assessing the inhibitory regulation of adenylyl cyclase. It is evident from the data shown in Fig. 3C that inhibition of forskolin-induced [3H]cAMP formation was observed over a reasonably comparable concentration range of the A1-receptor agonist CPA in cells expressing the A1-adenosine receptor (○ in Fig. 3C) and in cells expressing the fusion protein A1/Gαi,1 (● in Fig. 3C). Half-maximum inhibition was observed at 0.3 ± 0.1 and 0.6 ± 0.1 nM CPA for the A1-adenosine receptor and A1/Gαi,1, respectively. Taken together, these data indicate that the A1/Gαi,1 fusion protein is expressed as a functional receptor capable of regulating its typical second messenger pathway.

High Affinity Agonist Binding to the A1/Gαi,1 Fusion Protein—Given the close proximity of receptor and G protein in the A1/Gαi,1 fusion protein, a more rapid association rate of [125I]HPIA was to be expected, if the association of receptor and G protein were the rate-limiting step in the formation of the ternary complex. The experiments summarized in Fig. 4 and Table I show that this was not the case; the apparent (pseudo-first order) association (kapp) and dissociation (kad) rates of the agonist [125I]HPIA on membranes prepared from HEK293 cells stably expressing the human A1-adenosine receptor (○ in Fig. 4) or the A1/Gαi,1 fusion protein (● in Fig. 4) were comparable; accordingly the calculated association rate constants kapp were similar within the experimental error (Table I). We have, furthermore, ruled out that the energy requirements for formation (or break-up) of the ternary complex differed between the na-
cative A₁-adenosine receptor and the fusion protein A₁/Gα₁-1 by determining the kinetics of agonist binding over a temperature range from 10 °C to 30 °C. The apparent (pseudo-first order) association rates \( k_{\text{app}} \) (open symbols in Fig. 5, A and B) and the dissociation rates (closed symbols in Fig. 5, A and B) obtained in these experiments were used to generate Arrhenius plots for \([^{125}\text{I}]\)HPIA binding to the human A₁-adenosine receptor (Fig. 5A) and the A₁/Gα₁-1 fusion protein (Fig. 5B). The difference between \( k_{\text{app}} \) and \( k_{\text{off}} \) corrected for the ligand concentration yields the Arrhenius plot for the rate constant \( k_{\text{on}} \) of the forward reaction (Fig. 5C). It is evident that linear Arrhenius plots were obtained in all cases; more importantly, a comparison of the slopes of the lines calculated for the native receptor and the fusion protein A₁/Gα₁-1 shows that they are reasonably similar. Thus, the two proteins do not differ with respect to their temperature dependence of ternary complex formation.

Interaction of the A₁/Gα₁-1 Fusion Protein with βγ-Dimers—In the absence of βγ-dimers, the A₁-adenosine receptor interacts only poorly with Gα or Gα subunits and the affinity of the receptor is greatly enhanced by the presence of βγ-dimers (10). As mentioned above the fusion protein A₁/Gα₁-1 accumulated to fairly high levels (stable cell lines expressing the A₁-Gα₁-1 fusion protein (about 15%) was resistant to the action of pertussis toxin (data not shown). Hence, the level of A₁/Gα₁-1 expression in these experiments were used to generate Arrhenius plots for the association and dissociation of \([^{125}\text{I}]\)HPIA binding to membranes harboring the human A₁-adenosine receptor (A) or the A₁/Gα₁-1 fusion protein (B). The apparent (pseudo-first order) association \( k_{\text{app}} \) (open symbols) and dissociation \( k_{\text{off}} \) (closed symbols) rates of \([^{125}\text{I}]\)HPIA binding were measured as described in the legend to Fig. 4 and under “Experimental Procedures” at the indicated temperatures. Data points represent mean ± S.E. of three to five individual determinations. Panel C, the association rate constant \( k_{\text{on}} \) was estimated by subtracting the calculated \( k_{\text{on}} \) values from the respective \( k_{\text{app}} \) values of panels A (●) and B (■) and by correcting this difference for the radioligand concentration (1 nM).

α-subunits are fused into a single molecule. The amino-terminus of the receptor is directly tethered to the receptor moiety in the fusion protein, the interaction with βγ-dimers may be sterically hindered. Pertussis toxin-catalyzed ADP-ribosylation of Gβ or Gγ is supported by βγ-dimers and the rate of the reaction depends on the interaction of α-subunits and βγ-dimers (24). We have exploited this property of the toxin to test if, in the intact cells, the fusion protein combines with βγ-dimers. As expected, incubation of cells expressing the human A₁-adenosine receptor with pertussis toxin led to a time-dependent loss of high affinity \([^{125}\text{I}]\)HPIA binding (C) in Fig. 6A; this was also seen in cells expressing the A₁/Gα₁ fusion protein (● in Fig. 6A). The differences between the two cell lines were modest. If the data were fitted to an equation describing a monoexponential decay, rate constants of 0.47 ± 0.1 and 0.68 ± 0.16 h⁻¹ were calculated for the A₁/Gα₁ fusion protein and the human A₁-adenosine receptor, respectively. It is also evident from Fig. 6 that there was a delay before the action of pertussis toxin was detectable. This hysteresis presumably resulted from the transmembrane permeation and activation of the toxin. Finally, a fraction of the A₁/Gα₁-1 fusion protein (about 15%) was resistant to the action of pertussis.
toxin. Access of the toxin to the carboxyl terminus of the G protein α-subunit may be sterically hindered by the presence of the receptor moiety. Regardless of the underlying reasons for these minor differences between fusion protein and wild-type receptor, it is safe to conclude that the bulk of the fusion protein interacted with βγ-subunits in the intact cells. Furthermore, the modest difference in the rate constants suggested that the affinity of the fused α-subunit moiety for βγ-dimers was somewhat reduced, but not dramatically compromised.

While these observations indicate that an interaction of the fusion protein with βγ-dimers is possible, they do not address the question if βγ-dimers are required to support ternary complex formation upon binding of an agonist to the fusion protein. This requirement can be seen for the native human A1-adenosine receptor (Fig. 6B; if membranes were prepared from pertussis-toxin-treated cells (in which high affinity [125I]HPIA binding was abolished), addition of Gαi1 (at a limiting concentration of 12.5 nM) did not reconstitute [125I]HPIA binding per se but required the addition of βγ-dimers to restore high affinity agonist binding (○ in Fig. 6A). Because this approach cannot be employed with the A1/Gαi1 fusion protein, we have generated membranes that were depleted of βγ-dimers (>90% as estimated by immunoblotting) by detergent extraction. Under these conditions about one-third of the fusion protein is retained in the particulate fraction (as assessed by [3H]DPCPX binding). Addition of βγ-dimers promoted high affinity agonist binding (● in Fig. 6B) and the half-maximum effect was seen at 22 ± 5 nM βγ-dimers; this affinity estimate is somewhat lower than that determined in parallel for the unfused A1-adenosine receptor (EC50 = 11 ± 3 nM). More importantly, these observations indicate that βγ-dimers are required for ternary complex formation by the A1/Gαi1 fusion protein.

Alterations in the Affinity of the Receptor for the G Protein Affects the Dissociation of the Ternary Complex—In order to test the hypothesis that the dissociation rate rather than the rate of the forward reaction is crucial for ternary complex formation, we have replaced cysteine 351 of Gαi1 by glycine or isoleucine. This substitution not only renders the G protein resistant to ADP-ribosylation by pertussis toxin but also yields G proteins with an altered affinity for their cognate receptors (29). These fusion proteins A1/Gαi1.C351G and A1/Gαi1.C351I were expressed in HEK293 cells. We stress that cells expressing these fusion proteins were always pretreated with pertussis toxin to prevent the receptor in the fusion protein from interacting with the G proteins endogenous to the membrane (30). Two approaches were used to estimate the affinity of the receptor for the mutated G protein moiety in the tandem. First, we have determined the ability of GDP to suppress the formation of the ternary complex. Because the activated receptor reduces the affinity of the G protein for GDP (by promoting GDP release), an excess of GDP conversely lowers the affinity of the G protein for the receptor (1). Fig. 7A shows the inhibition of high affinity [125I]HPIA binding to membranes prepared from HEK293 cells expressing the wild-type fusion protein A1/Gαi1 (●) as well as the mutated versions A1/Gαi1.C351I (○) and A1/Gαi1.C351G (Δ) by GDP. Half-maximum inhibition by GDP was observed at 5.01 ± 1.55 μM for A1/Gαi1, 1.44 ± 0.35 μM for A1/Gαi1.C351I, and 0.39 ± 0.16 μM for A1/Gαi1.C351G. The second approach relied on the use of suramin. This compound binds directly to G protein α-subunits (31) and competes with the activated receptor for binding to the G protein; suramin can therefore be employed to estimate the affinity of a receptor for a G protein (21, 32). High affinity agonist binding to A1/Gαi1 fusion proteins mutated at Cys351 of the Gαi1 moiety was more readily suppressed by suramin (Fig. 7B). The IC50 of suramin was 8.55 ± 2.2 μM for A1/Gαi1 (● in Fig. 7B), 2.99 ± 0.67 μM for A1/Gαi1.C351I (○ in Fig. 7B), and 1.02 ± 0.06 μM for A1/Gαi1.C351G (Δ).

Based on the data summarized in Fig. 7, we concluded that Gαi1.C351I exhibited the lowest affinity for the A1-adenosine receptor; therefore the kinetics of ternary complex formation of the fusion protein A1/Gαi1.C351G were investigated in detail. The mutation did not affect the forward reaction (Δ in Fig. 8A), the calculated koff being comparable within experimental error to that seen in the fusion protein containing the wild type version of Gαi1 (Table I); in contrast, the ternary complex formed by A1/Gαi1.C351G dissociated more readily (Δ in Fig. 8B). The koff of A1/Gαi1.C351G was about five times faster than that determined for the fusion protein A1/Gαi1 (Table I) and thus in reasonable agreement with the estimated difference in affinity obtained by the approaches in Fig. 7. The reduced stability of the ternary complex was seen over the entire temperature range investigated for A1/Gαi1.C351G (Δ in Fig. 8C) and was also seen in the fusion protein A1/Gαi1.C351I (○ in Fig. 8C).


FIG. 7. Inhibition of agonist radioligand binding $[^{125}\text{I}]$HPIA to membranes prepared from HEK293 cells expressing $A_1/G_{\alpha_{i-1}}$ (●), $A_1/G_{\alpha_{i-1}C351I}$ (○), or $A_1/G_{i-1}C351G$ (△) by GDP (A) or suramin (B). The binding reaction was carried out in 40 μl containing membranes (2–10 μg of protein), and $[^{125}\text{I}]$HPIA (final concentration 1 nM) and increasing amounts of GDP (A) or suramin (B) for 90 min at 25 °C. Nonspecific binding was determined in the presence of 1 μM CPA. Specific binding in the absence of any compound (1.5–2 fmol of ligand bound) was set 100%. Data are mean ± S.E. from three separate experiments carried out in duplicate.

FIG. 8. Association (panel A) and dissociation (panel B) kinetics of $[^{125}\text{I}]$HPIA binding to membranes prepared from HEK293 cells stably expressing $A_1/G_{\alpha_{i-1}}$ (●) or $A_1/G_{i-1}C351G$ (△). The assay conditions were the same as outlined in the legend to Fig. 4; cells expressing $A_1/G_{i-1}C351G$ were first treated with 100 ng/ml pertussis toxin for 18 h before harvesting and membrane preparation. Data are means from three independent experiments carried out in duplicate with different membrane preparations and are expressed as percent of the respective equilibrium binding values ($B_{eq}$; error bars indicate S.E.). The $B_{eq}$ values amounted to $0.45 ± 0.07$ pmol/mg for the $A_1/G_{i-1}C351G$ and $1.1 ± 0.2$ pmol/mg for the $A_1/G_{\alpha_{i-1}}$ fusion protein. Panel C, the dissociation rate of $[^{125}\text{I}]$HPIA from $A_1/G_{i-1}C351G$ (△) and $A_1/G_{i-1}C351G$ (○) were determined at the indicated temperatures as in panel B. The values for $A_1/G_{\alpha_{i-1}}$ (●) were taken from Fig. 5A to illustrate the difference between the fusion protein containing a wild-type $G_{\alpha_{i-1}}$ moiety and those containing the mutated versions.

DISCUSSION

A fusion construct of the $\beta_2$-adrenergic receptor and $G_{\alpha_{i}}$ was originally shown to yield a functional protein capable of efficiently activating its prototypic effector adenyl cyclase (33, 34); this approach has more recently been extended to other receptor/G protein tandems and several aspects of receptor-G protein coupling have been investigated with these constructs (29, 35; for review, see Ref. 36). In the present work, we have used fused tandems of the $A_1$-adenosine receptor and G protein $\alpha$-subunits to test if the association of receptor and G protein is rate-limiting for ternary complex formation. It is evident from our experiments that the direct fusion of the receptor to the G protein $\alpha$-subunit does not accelerate the rate of ternary complex formation, although the local reactant concentration must, by definition, be very high. We rule out that the slow association rate is due to a lack of $\beta_\gamma$-dimers because overexpression of $\beta_\gamma$ does not affect the rate of the complex formation. Similarly, in reconstitution experiments, no appreciable acceleration in the rate of ternary complex formation was observed upon variation in the concentration of G protein oligomers. Our findings rather support the interpretation that the rate-limiting step(s) are those that govern the transition from the inactive to the active conformation of the receptor (3). In fact, in the absence of a G protein, the rate of agonist binding to the purified $\beta_2$-adrenergic receptor has been inferred from the alterations in fluorescence of appropriately labeled receptors; the rate of fluorescence change was also too slow to be accounted for by a simple diffusion-controlled reaction and was therefore proposed to reflect the rate-limiting change in conformation to the active species HR$^*$ (37). Finally, fusing the receptor and G protein moiety did not lower the activation energy required for complex formation; this is also consistent with the interpretation that the interaction of receptor and G protein is not rate-limiting. For both, the $A_1$-adenosine receptor and the fusion protein $A_1/G_{\alpha_{i-1}}$, the activation energies $E_a$ were estimated in the range of 45 kJ mol$^{-1}$. While this value is higher than that reported for the $\alpha_2$-adrenergic receptor in human platelet membranes (38), it is comparable to estimates that can be calculated from kinetic data that have been reported for high affinity agonist binding to the $A_1$-adenosine receptor in brain membranes (39).

Our interpretation relies on two simplifications: (i) we assume the rate of $[^{125}\text{I}]$HPIA dissociation is limited by the stability of the interaction between $R^*$ and $G$; this assumption is justified for a high affinity agonist because the affinity of $R^*$ for
Ga, βγ is lower than that for [125I]HIAPA. The apparent affinity of the human A₁-adenosine receptor in HEK 293 membranes for Ga, βγ has previously been estimated by two independent methods and is in the range of 10 nM (21); this value is also consistent with the affinity estimate obtained in reconstitution experiments (12). In contrast the KD for high affinity binding of [125I]HIAPA is about 1 nM. (ii) Ternary complex formation is treated as a bimolecular reaction; because the rate of ternary complex formation is independent of G protein concentration (i.e., not limited by the association of the receptor with the G protein), we assume the transition of the inactive conformation R to R* as the rate-limiting step. The agonist may stabilize R* by shifting the spontaneous equilibrium between R and R* or induce the formation of R* (3); regardless of which of these two models is applicable, ternary complex formation can be treated as the product of a bimolecular reaction of R* and G.

It has long been appreciated that the carboxyl terminus of Ga-subunits is important for binding of receptors; accordingly, mutations that substitute the cysteine residue at the carboxyl-terminal position –4 are expected to affect the affinity of the G protein for the receptor (16, 17). This has recently been systematically investigated by examining the interaction of the α2A-adrenergic receptor with Ga, in which this residue was replaced by the other 19 naturally occurring amino acids (29). While substitution with glycine reduces the affinity of both, the A₁-adenosine and the α2A-adrenergic receptor, it is worth noting that the replacement by isoleucine has different effects; it increases the affinity of the mutated G protein moiety in the α2A-adrenergic receptor/Ga₁ fusion protein (29) but reduces the affinity in the A₁/Ga₁ tandem. Analogous discrepancies have been noted in related studies. Some, but not all, G- coupled receptors can couple to mutated forms of Ga in which the last 5 amino acids were replaced with the corresponding residues of Ga₄; the same is true for G-coupled receptors that are confronted with a carboxyl-terminal altered Ga (40). Similarly, peptides derived from the carboxyl terminus of the cognate G protein α-subunits are capable of stabilizing the receptor in the conformation that binds agonists with high affinity; this can be seen with some, but not all, receptors (41, 42). Taken together, these findings highlight the different modes by which receptors engage the same G protein α-subunit and support the concept that the contact site is different enough in individual receptor-G protein complexes to allow this site to be considered as a potential target for inhibitors (32, 43).

The ability of a given receptor to engage a G protein may be limited by its ability to associate with the appropriate G protein; alternatively, the activated receptor may form complexes with various types of G proteins but only those complexes that dissociate slowly are stabilized to support efficient signaling. In order to differentiate between these two possibilities, we have created fusion proteins in which the affinity of the receptor for the G protein was lowered by substituting the critical cysteine residue with glycine or isoleucine. Our data clearly show that the rate of ternary complex formation is not affected by lowering the affinity of the G protein for the receptor; in contrast, the ternary complex dissociates more rapidly. These findings provide an explanation for the fidelity of signaling that is usually observed; they also account for the observation that upon overexpression of receptors the fidelity is lost; i.e., overexpressed receptors have the propensity to interact with G proteins and activate downstream signaling pathways that are not subject to their physiological regulation (reviewed in Ref. 6). Under these conditions, low concentrations of agonists typically suffice to promote the interaction of the receptor with the cognate G protein; however, high concentrations of agonists, i.e., in excess of those necessary to saturate the receptor, typically result in activation of one or more additional G proteins and their downstream signaling pathways. Our observations indicate that this high agonist concentration is required to increase the lifetime of the ternary complex resulting from the interaction of the receptor with the non-physiological G protein(s). In contrast, at low agonist concentrations, the “strength of signal” (6) does not suffice, because the lifetime of these ternary complexes is too short to support signal transduction. This model also predicts that a partial agonist is converted to an antagonist, if the affinity of the G protein for the receptor is lowered. This has indeed been recently observed; the intrinsic activity of the partial agonist clonidine is absent in a fusion protein composed of the α2A-adrenergic receptor and Ga₄G351C and the compound acts as an antagonist (44). Thus, the different dissociation rates of the ternary complex allow for a kinetic proofreading mechanism; the activated receptor can associate with various G proteins but only the cognate G protein(s) are retained in stable ternary complexes. Obviously, in intact cells additional factors contribute to the specific interaction of receptors and G proteins because the signaling molecules are compartmentalized. Receptors, for instance, are not uniformly distributed over the membrane in polarized cells (45) and in neurons (46) and components of the cytoskeleton and additional proteins are involved in the organization of G proteins and effectors (47, 48). Nevertheless, we propose that kinetic proofreading is important for a receptor to faithfully select its cognate partner(s) from the total G protein pool present in its vicinity.

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REFERENCES
1. Hepler, J., and Gilman, A. G. (1992) Trends Biochem. Sci. 17, 383–387
2. Neubig, R. R., Gantzos, R. D., and Thomsen, W. J. (1988) Biochemistry 27, 2374–2384
3. Gether, U., and Kohlika, B. K. (1988) J. Biol. Chem. 273, 19779–19782
4. Schütz, W., and Frei, M. (1992) Trends Pharmacol. Sci. 13, 376–380
5. Letkowitz, R. J., Cotecechia, S., Samam, P., and Costa, T. (1993) Trends Pharmacol. Sci. 14, 303–307
6. Kenakin, T. (1996) Pharmacol. Rev. 48, 413–463
7. Thomsen, W. J., and Neubig, R. R. (1989) Biochemistry 28, 8778–8786
8. Flocker, S., Breuer, H. P., Petraccione, F., Honerkamp, J., and Hofmann, K. P. (1990) Biochem. Biophys. Res. Commun. 170, 3051–3063
9. Gudermann, T., Ströer, M., and Schütz, G. (1997) Annu. Rev. Neurosci. 20, 383–427
10. Freissmuth, M., Schütz, W., and Linder, M. E. (1991) J. Biol. Chem. 266, 17778–17783
11. Monal, F., Pich, J. H., Sternweis, P. C., and Linden, J. (1991) J. Biol. Chem. 266, 22285–22299
12. Jockers, R., Linder, M. E., Hohenegger, M., Nanoff, C., Bertin, B., Strosberg, A. D., Arulparu, S., and Freissmuth, M. (1994) J. Biol. Chem. 269, 32077–32084
13. Figler, R. A., Graber, S. G., Linderber, M. A., Yasuda, H., Linden, J., and Garrison, J. C. (1996) Mol. Pharmacol. 50, 1587–1595
14. Nanoff, C., Roka, Mitterauer, S., Hohenegger, M., and Freissmuth, M. (1995) Mol. Pharmacol. 48, 806–817
15. Gao, Z., Roche, A. S., and Linden, J. (1999) J. Biol. Chem. 274, 729–736
16. Hess, W. (1997) PNAS 11, 346–354
17. Hämmer, H. H. (1998) J. Biol. Chem. 273, 669–672
18. Linden, J. (1984) Mol. Pharmacol. 26, 414–423
19. Freund, S., Unegger, M., and Lohse, M. J. (1994) Naunyn Schmiechen's Arch. Pharmacol. 350, 49–56
20. Hode, A., Sheehan, M., Res, S., Lee, M., and Milligan, G. (1999) Biochemistry 38, 2272–2278
21. Waldhofer, M., Bossi-Ford, M., Milligan, G., Freissmuth, M., and Nanoff, C. (1998) Mol. Pharmacol. 53, 808–818
22. Mumber, S. M., and Linder, M. E. (1994) Methods Enzymol. 257, 354–368
23. Nanoff, C., Waldhofer, M., Roka, M., and Freissmuth, M. (1997) Neuropharmacology 36, 1211–1219
24. Casey, J. P., Graziano, M. P., and Gilman, A. G. (1989) Biochemistry 28, 611–616
25. Schotten, E., Witting, A., Schiferer, E., Herrmann, M., Grubeck-Loebenstein, B., and Freissmuth, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1609–1613
26. Geogos, C., Zarr, C., and Milligan, G. (1993) Mol. Pharmacol. 44, 62–69
27. Johnson, R. A., and Salmon, Y. (1991) Methods Enzymol. 193, 33–41
28. Nakata, H. (1999) J. Biol. Chem. 274, 671–677
29. Bahia, D. S., Wise, A., Fanelli, F., Lode, M., Rees, S., and Milligan, G. (1998) Biochemistry 37, 11555–11562
30. Burt, A. R., Small, M., Wilson, J. M., Rees, S., and Wise, A., and Milligan, G.
31. Freissmuth, M., Boehm, S., Heindl, W., Nickel, P., Ilzerman, A. P., Hohenegger, M., and Nanoff, C. (1996) *Mol. Pharmacol.* **49**, 602–611
32. Freissmuth, M., Waldhoer, M., Bofill-Cardona, E., and Nanoff, C. (1999) *Trends Pharmacol. Sciences* **20**, 237–245
33. Bertin, B., Freissmuth, M., Jockers, R., Strousberg, A. D., and Marullo, S. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 8827–31
34. Seifert, R., Lee, T. W., Lam, V. T., and Kobilka, B. K. (1998) *Eur. J. Biochem.* **255**, 369–382
35. Wise, A., and Milligan, G. (1997) *J. Biol. Chem.* **272**, 24673–24678
36. Seifert, R., Wenzel-Seifert, K., and Kobilka, B. K. (1999) *Trends Pharmacol. Sci.*, **20**, 383–389
37. Gether, U., Lin, S., and Kobilka, B. K. (1995-96) *J. Biol. Chem.* **270**, 28268–28275
38. Gantzos, R. D., and Neubig, R. R. (1988) *Biochem. Pharmacol.* **37**, 2815–2821
39. Casado, V., Allende, G., Mallol, J., Franco, R., Lluis, C., and Canela, E. I. (1993) *J. Pharmacol. Exp. Ther.* **266**, 1463–1474
40. Conklin, B. R., Herzmark, P., Ishida, S., Veyno-Yasenetskaya, T. A., Sun, Y., Farfel, Z., and Bourne, H. R. (1996) *Mol. Pharmacol.* **50**, 885–890
41. Rasenick, M. M., Watanabe, M., Lazarevic, M. B., Hatta, S., and Hamm, H. E. (1994) *J. Biol. Chem.* **269**, 21519–21525
42. Gilchrist, A., Mazzoni, M. R., Dineen, B., Dice, A., Linden, J., Proctor, W. R., Lupica, C. R., Dunwiddie, T. V., and Hamm, H. E. (1998) *J. Biol. Chem.* **273**, 14912–14919
43. Hoeller, C., Freissmuth, M., and Nanoff, C. (1999) *Cell. Mol. Life Sci.* **55**, 257–270
44. Jackson, V. N., Bahia, D. S., and Milligan, G. (1999) *Mol. Pharmacol.* **55**, 195–201
45. Saunders, C., Keefer, J. R., Kennedy, A. P., Wells, J. N., and Limbird, E. (1996) *J. Biol. Chem.* **271**, 995–1002
46. Tu, J. C., Xiao, B., Yuan, J. P., Lanahan, A. A., Leoffert, K., Li, M., Linden, D. J., and Worley, P. F. (1996) *Neuron* **21**, 717–726
47. Neubig, R. R. (1994) *FASEB* **8**, 939–945
48. Huang, C., Hepler, J. R., Chen, L. T., Gilman, A. G., Anderson, R. G. W., and Mumby, S. M. (1997) *Mol. Biol. Cell* **8**, 2365–2378

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