Fluorescence Analysis of Receptor−G Protein Interactions in Cell Membranes[†]

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ABSTRACT: The dynamics of G protein heterotrimer complex formation and disassembly in response to nucleotide binding and receptor activation govern the rate of responses to external stimuli. We use a novel flow cytometry approach to study the effects of lipid modification, isoform specificity, lipid environment, and receptor stimulation on the affinity and kinetics of G protein subunit binding. Fluorescein-labeled myristoylated $G\alpha_{i1}$ (F-α_{i1}) was used as the ligand bound to $G\beta\gamma$ in competition binding studies with differently modified $G\alpha$ subunit isoforms. In detergent solutions, the binding affinity of $G\alpha_i$ to $\beta\gamma$ was 2 orders of magnitude higher than for $G\alpha_o$ and $G\alpha_s$ (IC_{50} of 0.2 nM vs 17 and 27 nM, respectively), while in reconstituted bovine brain lipid vesicles, binding was slightly weaker. The effects of receptor on the G protein complex were assessed in α_{2A}AR receptor expressing CHO cell membranes into which purified $\beta\gamma$ subunits and F-α_{i1} were reconstituted. These cell membrane studies led to the following observations: (1) binding of α subunit to the $\beta\gamma$ was not enhanced by receptor in the presence or absence of agonist, indicating that $\beta\gamma$ contributed essentially all of the binding energy for α_{i1} interaction with the membrane; (2) activation of the receptor facilitated GTPγS-stimulated detachment of F-α_{i1} from $\beta\gamma$ and the membrane. Thus flow cytometry permits quantiatitive and real-time assessments of protein−protein interactions in complex membrane environments.

In mammals, the diverse family of seven transmembrane receptors represents a primary cellular target for intercellular signaling. The cycle of formation, reversible interaction, and disengagement of the signal transducing protein machinery governs the cascade of intracellular responses (*1*). Activation of a receptor triggers nucleotide exchange on the preassembled G protein $\alpha-\beta\gamma$ heterotrimer on the inner surface of cell membrane. The intrinsic GTPase activity of $G\alpha$ provides a return mechanism to the initial heterotrimeric arrangement of G protein on the surface of the membrane. Characterization of key modulators of receptor−G protein signaling including G protein isoform specificity, lipid modification, cell membrane environment, and nucleotide selectivity is important to fully understand this system.

The G protein β and γ subunits form a functional unit that can be divided only under denaturing conditions. The $G\beta\gamma$ dimers are highly hydrophobic and in vivo are confined exclusively to the membrane. The lateral mobility of $G\beta\gamma$ in the membrane leading to stochastic collision with a receptor is a hypothesized mechanism of receptor-mediated G protein activation (*2*) though clustered aggregate formation has also been proposed (*3*−*6*). N-Terminal myristoylation of $G\alpha$ isoforms (α_{i1}, α_{i2}, α_{i3}, α_o, α_z, and α_t) has been proposed to anchor them to the plasma membrane (*7*, *8*). From more recent observations it appears that myristoylation alone may not be sufficient for the membrane association. Dynamic palmitoylation is thought to direct $G\alpha$ to the membrane, where two fatty acid chains form a hydrophobic clip that is inserted in the plasma membrane matrix (*9*, *10*). In the case of $G\alpha_s$, which is not myristoylated, prolonged agonist exposure of the β_2AR receptor results in depalmitoylation and is thought to lead to its translocation to the cytosol (*11*).

While the molecular basis for receptor−$G\alpha\beta\gamma$ protein interaction has been unraveled in significant detail, the topological organization and mechanistic aspects of ternary complex function in the cell membrane are still in need of further clarification. Reconstitution of the receptor−G protein signaling pathways has become a useful tool for controlled analysis of the dynamics of the interaction, receptor-dependent nucleotide binding to the G protein, and conformational changes that accompany activation (*12*−*15*). In this study we applied a flow cytometry approach to study high-affinity interactions in multiprotein complexes to quantitate the effects of lipid modification of $G\alpha$ isoforms on their coupling to the $\beta\gamma$ subunits in different environments. In addition, we investigated the role of α_2AR[1] receptor coupling and ternary complex formation on the G protein $\alpha-\beta\gamma$ subunit interactions in two cell membranes. Effects of G protein activation on $G-\alpha_{i1}$ membrane association were examined by real-time measurements of fluorescently labeled F-α_{i1} release from the ternary complex in the cell membrane.

EXPERIMENTAL PROCEDURES

Materials. N-[6-(Biotinoylamino)hexanoyl]dipalmitoyl-L-α-phosphatidylethanolamine triethylammonium salt (EZ-Link biotin-LC-DPPE) was obtained from Pierce (Rockford, IL).

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Biotinamidocaproate *N*-hydroxysuccimide ester (BXNHS), ω-aminobutylagarose, and bovine brain membrane extract (catalog no. B3635) were purchased from Sigma Chemical Co. (St. Louis, MO). Biotinylated concanavalin A was obtained from EZ Laboratories (San Mateo, CA). Fluorescein 5-isothiocyanate (FITC) was obtained from Molecular Probes, Inc. (Eugene, OR). Fluorescein-labeled anti-hemaglutinin (HA) epitope antibodies were from Boehringer Mannheim (Indianapolis, IN). Streptavidin-coated 6 μm polystyrene beads were purchased from Spherotech, Inc. (Liberville, IL). Other reagents were from standard suppliers.

Purification and Labeling of G Protein Subunits. The expression vectors pQE6 containing rat cDNA sequence for $G_i\alpha_1$ and pBB131 containing yeast *N*-myristoyltransferase were generously provided by Drs. Maurine Linder and Jeffrey Gordon, respectively (Washington University, St. Louis, MO). Recombinant myristoylated G protein α_{i1} (r-myr-α_{i1}) was expressed in *Escherichia coli* and purified according to ref *16* with minor modifications. Bovine brain G$\beta\gamma$ subunits were isolated from purified G_o/G_i as described (*17*). Purified r-myr-α_{i1} was labeled with fluorescein isothiocyanate (FITC) as described in detail (*18*). After affinity isolation on a $\beta\gamma$-agarose column, the fluorescein-labeled r-myr-α_{i1} (F-α_{i1}) had a specific activity of 11 pmol/μg of [^{35}S]GTPγS binding and stoichiometry of dye to protein of 0.9. Recombinant α_s and myristolyated α_o were expressed in *E. coli* and were kind gifts of Dr. Ron Taussig (University of Michigan). His_6-tagged Gα_o and α_{i1} had amino-terminal His_6 tags and were purified as described (*19*).

The G protein heterotrimer was biotinylated by the amino group-specific derivative BXNHS (biotinamidocaproate *N*-hydroxysuccimide ester) as described (*20*, *21*). Biotinylated $\beta\gamma$ (b-$\beta\gamma$) subunits were isolated through the activation of the biotinylated heterotrimer by 10 mM $MgCl_2$, 10 mM NaF, and 50 μM $AlCl_3$ followed by several steps of column chromatography (*18*). The resulting b-$\beta\gamma$ was 99% pure by Coomassie Blue staining on SDS−PAGE, and the β subunit contained all of the biotin detectable by Western blot analysis with peroxidase-labeled avidin.

Reconstitution of $\beta\gamma$ in Lipid Velicles. To attach $\beta\gamma$ to beads while maintaining a lipid environment, phospholipid vesicles containing 1% biotin−lipid were prepared from bovine brain membrane extract and purified $\beta\gamma$ subunits according to methods previously described (*22*, *23*). EZ-Link biotin-LC-DPPE (biotin−lipid), featuring a 2.7 nm spacer arm to avoid steric hindrance, was mixed in a 1:100 molar ratio with brain lipids to permit attachment of the vesicles to the avidin-coated beads (*18*). Phosphatidylcholine or bovine brain membrane extract (0.8 mg) was mixed with 8 μg of biotin−lipid in chloroform−methanol (2:0), dried under N_2, and resuspended in 0.2 mL of a 1.2% solution of sodium cholate. Lipids were mixed with purified $\beta\gamma$ in 1.2% cholate at a 100:1 lipid to protein molar ratio; the mixture was vortexed for 5 min at room temperature and sonicated for 30 min at 4 °C in Branson 1200 sonicator. Vesicles were prepared by gel filtration through a Sephadex G 25-50 column equilibrated with 50 mM HEPES, 1 mM EDTA, 1 mM DTT, pH 8.0, buffer. Turbid fractions containing $\beta\gamma$ were pooled, snap-frozen in aliquots in liquid nitrogen, and stored at −80 °C. The relative amount of $\beta\gamma$ incorporated in vesicles was estimated by immunostaining with β-specific antibody (MS/1, amino-terminal β-specific rabbit antisera; NEN Life Science Products), followed by staining with secondary FITC-conjugated antibody and detection by flow cytometry.

Preparation of Biotin-Labeled Sf9 Plasma Membrane Vesicles. The α_2-adrenergic receptor (α_2AR) and/or β_1 and γ_2 subunits of G protein were expressed using a baculovirus expression system (*24*) and plasma membranes purified as described by Sarvazyan and Neubig (*25*). Sf9 (*Spodoptera frugiperda*) cells were infected at $(1-2) \times 10^6$/mL density with the appropriate virus at a multiplicity of infection (MOI) of 1 and cultured in serum-free Grace's medium. Infected cells were harvested intact between 48 and 52 h after infection by centrifugation for 5 min at 500*g* and washed twice with gentle resuspension in $^1/_{10}$ of the original volume of ice-cold 20 mM Tris, pH 7.6, 150 mM NaCl, 1 mM EDTA (TNE) buffer. Biotinylated concanvalin A lectin (b-Con-A) was added to the cell suspension in the presence of 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The mixture was incubated for 20 min at room temperature, diluted in TNE buffer, and washed twice by centrifugation for 5 min at 500*g*. All subsequent steps in the membrane preparation were done at 0−4 °C. Cells were lysed in hypotonic buffer (20 mM Tris, pH 8.0, 1 mM EDTA, 100 μM PMSF, 2 μg/mL aprotinin, and 10 μg/mL leupeptin) followed by a nitrogen cavitation for 20 min at 600 psi. Cell membranes were further disrupted by a 30 min sonication in a Branson 1200 sonicator water bath. Unbroken cells and nuclei were removed by centrifugation at 1000*g* for 10 min. The supernatant was subjected to centrifugation at 10000*g* for 30 min, which separated the intracellular light membranes in the supernatant and a heavy plasma membrane in the pellet (*25*). The pellet was homogenized by 10 strokes in a glass−Teflon homogenizer and layered on top of 10 mL of 15% Percoll (Pharmacia Biotech, Uppsala, Sweden) in 10 mM Tris, pH 7.6, 1 mM EDTA, 1 mM DTT, and 250 mM sucrose buffer. The membrane fractions were separated by centrifugation for 25 min at 24000*g* in an angle-head 65Ti rotor. A balance tube containing density marker beads was used to monitor the density of the in situ formed continuous Percoll gradient. Fractions (0.6 mL) were collected from the top of the Percoll gradient and analyzed by Bradford protein assay (*27*) and by [^{3}H]yohimbine binding for the α_2AR receptor as a plasma membrane marker. From the two distinct turbid fractions collected, the upper lighter membrane fraction was collected as described (*25*).

Assembly of the R−G Protein Complex in CHO Membranes. A stable CHO cell line expressing high levels of an HA epitope-tagged α_2AR receptor (Tag L1, 20−40 pmol of [^{3}H]yohimbine binding/mg of protein) was isolated by cell sorting as previously described (*28*, *29*). Membranes were

[1] Abbreviations: α_2AR, α_2-adrenergic receptor; b-$\beta\gamma$, biotinylated G protein $\beta\gamma$ subunits; biotin-LC-DPPE, *N*-[6-(biotinoylamino)hexanoyl]dipalmitoyl-L-α-phosphatidylethanolamine triethylammonium salt; BXNHS, biotinamidocaproate *N*-hydroxysuccimide ester; CHO, Chinester hamster ovary cells; F-α_{i1}, FITC-labeled G protein α_{i1} subunit; FITC, fluorescein 5-isothiocyanate; GTPγS, guanosine 5′-(γ-thio)triphosphate; HEDNM(BSA), 20 mM HEPES, 1 mM EDTA, 1 mM DTT, 150 nM NaCl, 0.2 mM free Mg^{2+}, and 0.1% BSA; HEDNML buffer, 20 mM HEPES, 1 mM EDTA, 1 mM DTT, 150 nM NaCl, 0.2 mM free Mg^{2+}, and 0.1% Lubrol; HA, hemaglutinin; PIC, *p*-iodoclonidine; r-myr-α_{i1}, recombinant myristoylated G protein α_{i1}; TNE buffer, 20 mM Tris, pH 7.6, 150 mM NaCl, and 1 mM EDTA; TME buffer, 50 mM Tris, 5 mM $MgCl_2$, and 1 mM EDTA.

prepared as previously described (*28*) except that the final membrane pellets were gently resuspended in TNE (20 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.6). Portions of the membranes were also treated with urea to inactivate G protein subunits as described (*30*−*32*). Biotinylated concanvalin A lectin (b-Con-A) was added to the urea-treated or control membranes in the presence of 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The mixture was incubated for 20 min at room temperature, diluted with the TNE buffer, and washed two times by centrifugation for 30 min at 150000*g* in a Beckman 65Ti rotor. Labeled membranes were resuspended in TNE buffer with 1 mM DTT added, snap-frozen, and stored at −80 °C before use.

Purified bovine brain $\beta\gamma$ subunits were reconstituted with the b-Con-A labeled, urea-treated CHO cell membranes by dilution from 0.1% sodium cholate solution. Specifically, a 40 μL volume of TNE containing 0.1% cholate, 40 μg of CHO membranes, and 4 pmol of $\beta\gamma$ was vortexed, incubated at room temperature for 10 min with shaking, and then diluted to 1 mL in HEDNM buffer containing 10 μM GDP and 0.1% BSA.

Preparation of Cell Membrane-Covered Beads for Flow Cytometry Analysis. Ten micrograms of the biotin−Con-A-treated membranes from either the Sf9 membrane light fraction or urea-treated CHO cell membranes was incubated with 10^5 avidin-coated beads in 200 μL of HEDNM, 10 μM GDP, 0.1% BSA buffer for 20 min. Samples were washed to remove unbound membranes by centrifugation in an Eppendorf centrifuge for 2 min at 10000 rpm and then resuspended in a final 1 mL volume of the same buffer.

Flow Cytometric Analysis of Equilibrium F-α_{i1} Binding. Flow cytometric analyses of G protein subunit interactions were performed on a Becton-Dickinson FACScan as described (*18*). For binding of F-α_{i1} to $\beta\gamma$ in detergent solution, avidin-coated beads were incubated with the biotin-$\beta\gamma$ in HEDNML (20 mM HEPES, 1 mM EDTA, 1 mM DTT, 150 nM NaCl, 0.2 mM free Mg^{2+}, 0.1% Lubrol). For binding of F-α_{i1} to $\beta\gamma$ in lipid vesicles and cell membranes, the biotinylated samples were bound to the beads in HEDNM-(BSA) solution (20 mM HEPES, 1 mM EDTA, 1 mM DTT, 150 nM NaCl, 0.2 mM free Mg^{2+}, 0.1% BSA). Preparation of beads was done within 3 h of each binding experiment. After 20 min incubation at room temperature (21−23 °C) the beads were washed twice by sedimentation in an Eppendorf centrifuge to remove unbound material. Samples were diluted in the appropriate buffer so that the final density of beads was 10^5/mL and the indicated concentration of proteins was reached. In equilibrium binding experiments, 0.1−50 nM F-α_{i1} was incubated in a 0.1 mL reaction volume with 10^5 beads/mL for 30 min at room temperature in the dark. In competition binding experiments, the indicated amounts of competing Gα isoforms were mixed with 0.5 nM F-α_{i1} and then incubated with 0.05 nM $\beta\gamma$ subunits precoupled to the avidin beads. Nonspecific binding was determined by addition of a 50-fold excess of unlabeled r-myr-α_{i1} subunit over F-α_{i1}. Data were collected on the forward scatter (FSC), side scatter (SSC), and fluorescein (FL-1) channels. The fluorescence intensity signal (mean channel number) from the singlet population of 1000−5000 beads was measured.

Flow Cytometric Analysis of F-α_{i1} Association and Dissociation Kinetics. Dissociation of 1 nM F-α_{i1} from 0.4 nM $\beta\gamma$ reconstituted in receptor-containing CHO membranes was monitored as a time-dependent decrease in mean fluorescence channel (FL-1). Dissociation of the subunits was initiated by addition of a small volume of unlabeled r-myr-α_{i1} subunit to obtain a 50-fold excess over F-α_{i1}. Alternatively, in the activation-induced dissociation experiments, the reaction was initiated by addition of 2 μL of 100 μM GTPγS to 400 μL of preassembled heterotrimer. The larger volume of sample in kinetic measurements (400 μL) was required due to the longer duration of measurements. Two modes of time-resolved reading were utilized: continuous and discontinuous. In continuous mode, each sample was measured without interruption with the changes in intensity of fluorescence monitored continuously over time with second time resolution. In discontinuous mode, a series of individual readings were performed at different time points following initiation of the reaction. In discontinuous mode, the average fluorescence from 500 to 1000 events was recorded within 5 s of the indicated time point. While more laborious, the discontinuous measurements offered higher sensitivity and were more applicable for longer time intervals.

Radioligand Binding Assay. To reconstitute high-affinity [^{125}I]PIC binding, urea-treated, α_{2A}AR-expressing Sf9 membrane suspensions were incubated with myristoylated α_{i1} and bovine brain $\beta\gamma$ at a molar ratio of receptor:α:$\beta\gamma$ of 1:25:80 in 120 μL of buffer containing TME buffer (50 mM Tris, 5 mM $MgCl_2$, 1 mM EDTA) supplemented with 1 mM DTT and 0.1% cholate, pH 7.6. Samples were vortexed and kept on ice for 1 h prior to a 5-fold dilution into TME buffer.

[^{3}H]Yohimbine and [^{125}I]PIC binding was measured in 96-well plates with 0.4 μg of protein per well in a final volume of 100 μL as previously described (*33*, *34*). After incubation at room temperature for 60 min, samples were filtered through GF/C filters using a Brandel cell harvester. Filters were washed twice by 2 mL of TM (50 mM Tris, 5 mM $MgCl_2$) and air-dried, and radioactivity was determined by liquid scintillation counting. Nonspecific binding was defined by 10 μM yohimbine or oxymetazoline, respectively, and was less than 10% of total binding.

Data Analysis and Statistics. Binding parameters were determined from equilibrium data fit to a one-site hyperbolic binding function $[LR] = B_{max}[L]/(K_d + [L])$. Competition binding curves were fit by a one-site competition function. Dissociation kinetics were approximated with a single-exponential function. Prism (Graphpad Software, San Diego, CA) was used for unweighted nonlinear least-squares fitting of all of the data.

RESULTS

Flow cytometry is a sensitive and relatively simple technique commonly used to monitor or sort individual cells on the basis of physical properties and the binding of fluorescently labeled antibodies. We previously described the use of this methodology to study protein−protein interactions in which the "receptor" (which could be a pure protein or a protein bound on lipid vesicles or membrane fragments) is attached to cell-sized beads, then a fluorescently labeled ligand is allowed to bind to the receptor-containing beads, and the resulting fluorescence signal is measured (*18*, *25*, *35*). In the present study, we measured the extent and time course of fluorescently labeled Gα subunit binding to and

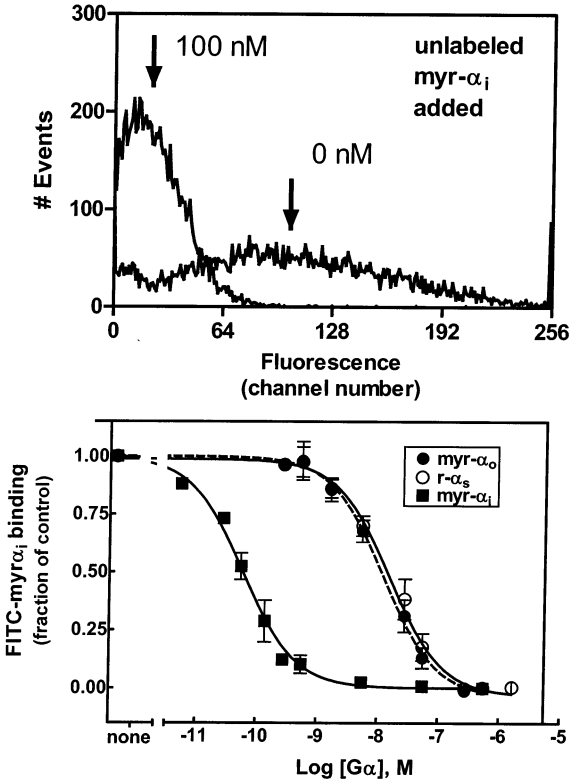

FIGURE 1: Competition binding of Gα isoforms to biotinylated b-$\beta\gamma$. Biotinylated b-$\beta\gamma$ (0.05 nM) was coupled to the avidin-coated beads in HEDNML (0.1% Lubrol, 0.1% BSA) buffer as described in Experimental Procedures. (Top panel) F-α_{i1} (0.5 nM) was added with or without 100 nM unlabeled myr-α_{i1}, and after 30 min at room temperature, bead-bound fluorescence was measured on the singlet bead population by flow cytometry. Histograms of fluorescence are shown, with the arrows indicating the mean channel number (102.1 for control and 23.1 for 100 nM myr-α_{i1}). Binding to avidin beads with no $\beta\gamma$ subunit was slightly lower than that obtained with excess myr-α_{i1} (mean channel number 16.6). (Bottom panel) F-α_{i1} (0.5 nM) and the indicated concentrations of unlabeled recombinant myristoylated α_{i1} (■), α_s (○), or myristoylated-α_o (●) were simultaneously added to the b-$\beta\gamma$ attached to the beads. Nonspecific binding determined in the presence of excess myr-α_{i1} was subtracted, and data were normalized to the binding of 0.5 nM F-α_{i1} alone. Curves are nonlinear least-squares fits to a one-site competition function. Data are means ± SE of three experiments performed in duplicate.

release from G$\beta\gamma$ in different environments including receptor-expressing mammalian cell membranes.

Recombinant myristoylated F-α_{i1} (0.5 nM) binding to b-$\beta\gamma$ (0.05 nM) on beads in detergent provides a substantial specific binding signal at nanomolar concentrations (Figure 1 and ref *18*). Nonspecific binding, defined in the presence of excess of unlabeled Gα_{i1}, was no more than 12−20% of total binding. The affinities of the α_{i1}, α_s, and α_o isoforms of G protein were measured by competition with F-α_{i1} for the b-$\beta\gamma$ binding sites on the beads in 0.1% Lubrol detergent. As illustrated in Figure 1, the amount of bead-bound fluorescence from F-α_{i1} decreased with concentration of the unlabeled Gα. From the competition binding data, the affinity of unlabeled α_{i1} toward $\beta\gamma$ was somewhat higher (IC_{50} 0.2 nM) than what we observed earlier in the saturation binding experiments with F-α_{i1} (K_d 3 nM) (*18*).[2] Both bacterially expressed α_s and myristoylated α_o had 100-fold lower affinities toward b-$\beta\gamma$ than did α_{i1} (Table 1).

Lipid modification of G protein α subunits is critical for association with the cell membrane, subcellular localization,

Table 1: Effects of Amino-Terminal Modification of Gα Subunit Isoforms and Lipid Environment on the Affinity of Binding to $\beta\gamma$[a]

alpha subunit	assay condition	IC_{50} (nM) unmodified	myristoylated	His_6-modified
α_{i1}	Lubrol	177 ± 75 (3)	0.21 ± 0.1 (3)	5.6 ± 1 (3)
α_o	Lubrol	≥1000 (3)	17 ± 5 (3)	263 ± 75 (3)
α_s	Lubrol	27 ± 10 (5)	ND	ND
α_{i1}	brain lipid vesicles	328 ± 108 (3)	0.7 ± 0.35 (3)	104 ± 12 (2)

[a] IC_{50} values were determined from competition binding experiments, as shown in Figures 1 and 2. Measurements for the top three rows were done in 0.1% Lubrol, and that in the bottom row was done with $\beta\gamma$ reconstituted in brain phospholipid vesicles. Data are means ± SE for each group. Average IC_{50} values were determined from nonlinear least-squares fits to a one-site competition function. ND means not determined.

interaction with the $\beta\gamma$ complex, and receptor coupling (*36*, *37*), but there is little quantitative information about the contribution of lipid modifications to α−$\beta\gamma$ subunit interactions. We determined the IC_{50} for binding of three different recombinant preparations of Gα subunits (myristoylated, 6-histidine tagged, and nonmodified) to $\beta\gamma$ subunits in 0.1% Lubrol solution. There was a marked increase of affinity of both α_{i1} and α_o (ca. 1000-fold) toward $\beta\gamma$ upon myristoylation (Table 1). Attempts to chemically palmitoylate fluorescently labeled α_{i1} resulted in a only partially palmitoylated protein (palmitate/F-α_{i1} of ∼0.3−0.5) and did not show any detectable increase in α subunit binding to $\beta\gamma$ either in Lubrol or in brain phospholipid vesicles vs that of myr-α_{i1} alone (see Discussion). Interestingly, addition of an N-terminal His_6 moiety also increased the affinity of α_{i1} and α_o for $\beta\gamma$ (ca. 30-fold). To evaluate the contributions of the $\beta\gamma$ subunit and the lipid environment in the effect of myristoylation on the association of Gα and $\beta\gamma$, the $\beta\gamma$ was reconstituted into phospholipid vesicles as previously described (*30*). There was virtually no competable binding of F-α_{i1} to beads with biotin-labeled lipid vesicles alone (data not shown) but binding to $\beta\gamma$/lipid beads was robust (Figure 2B). There was a slight shift toward lower affinity of α_{i1} when $\beta\gamma$ was in the lipid system (Figure 2), but the effect of myristoylation was still strong (ca. 500-fold) while the enhancement of affinity by His_6 modification was much less striking (ca. 3-fold).[3]

We wished to explore the energetics and dynamics of α−$\beta\gamma$ receptor coupling in cell membranes with this flow cytometry approach. It is well-known that $\beta\gamma$ is essential for Gα subunit association with membranes; however, we wanted to determine the extent to which Gα subunit interactions with receptor contributed additional binding energy for membrane association. As proof of concept for studying F-α_{i1} binding to $\beta\gamma$ containing Sf9 membranes, we

[2] The increased affinity of α_{i1} observed in the competition binding studies reported here compared to the previous saturation binding studies with F-α_{i1} (*18*) may be due to an effect of the fluorescent modification of α_{i1} on $\beta\gamma$ binding. The affinity measured here is for the native protein competing for the binding of F-α_{i1}. As noted in Experimental Procedures, however, the FITC labeling did not seem to change any major functional properties of the protein.

[3] Unfortunately, attempts to evaluate the effect of palmitoylation were not successful due to difficulties in obtaining stoichiometrically palmitoylated Gα subunits and to the instability of the modification and degradation of the palmitate concurrent with the binding measurements in our experiments.

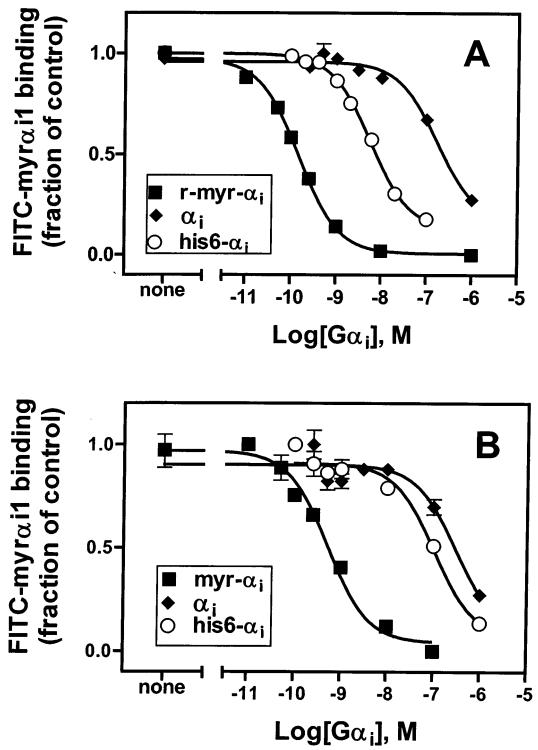

FIGURE 2: Effect of amino-terminal modification of the α_i subunit on binding to $\beta\gamma$ in detergent and in lipid vesicles. F-α_{i1} (0.5 nM) and the indicated concentrations of either α_i (◆), myr-α_i (■), or His_6-α_{i1} (○) were simultaneously added to the $\beta\gamma$ in detergent (A) or in lipid vesicles (B). Samples were incubated for 30 min at room temperature, and the fluorescence on the beads was measured by flow cytometry. (A) In detergent, biotinylated b-$\beta\gamma$ was coupled to the avidin-coated beads in HEDNML(BSA) buffer that contained 0.1% Lubrol. (B) In lipid vesicles, $\beta\gamma$ was reconstituted into phospholipid vesicles with 1% of the biotinylated LC-DPPE lipids incorporated. Biotinylated vesicles were coupled to the avidin-coated beads in HEDNM(BSA). Data were obtained in duplicates and are representative of three independent experiments. Curves are nonlinear least-squares fits to a one-site competition function.

prepared Sf9 membranes from cells infected either with a control baculovirus (lacZ) or with viruses for $\beta1$ and $\gamma2$ subunits ($\beta\gamma$). Preliminary studies with crude membrane preparations showed tremendous heterogeneity in the flow measurements so we isolated plasma membrane fractions on a Percoll gradient (*25*). These plasma membranes were then treated with biotinylated lectin that bound to endogenous Sf9 plasma membrane glycoproteins to link the membranes to avidin-coated beads. Equal amounts of control (lacZ) and $\beta\gamma$-expressing membranes bound to beads as demonstrated by staining with fluorescein-conjugated avidin (F-avidin) (Figure 3A). The expressed $\beta\gamma$ was accessible to the solution based on staining with an anti-β-subunit primary antibody followed by fluorescein-conjugated secondary antibody (Figure 3B). The control (lacZ) membranes showed equivalent amounts of membrane on the beads (F-avidin staining) but only a small amount of anti-β subunit staining. Also, the $\beta_1\gamma_2$ expressing membranes showed substantial specific binding of F-α_{i1} while the lacZ membranes showed only minimal specific F-α_{i1} binding. The residual binding of the F-α_{i1} to the control membranes may be due to the low level of endogenous $\beta\gamma$ expression or to low-affinity association of F-α_{i1} to the plasma membranes.

While the Sf9 membrane system showed the feasibility of studying $\alpha-\beta\gamma$ subunit interactions in cell membranes, we needed a higher level of receptor expression and better control of receptor/$\beta\gamma$ stoichiometry to study the effects of receptor on subunit interactions. Thus we turned to a stable CHO cell line expressing the porcine α_{2A}AR at levels up to 45 pmol/mg. This cell line was selected for very high receptor expression by flow cytometry using fluorescein-anti-HA staining as previously described (*28*, *36*). By reconstituting purified $\beta\gamma$ in the CHO cell membranes, we could readily manipulate the $\beta\gamma/\alpha_2$AR ratio in the membranes. To eliminate problems with heterogeneity of the endogenously expressed $\beta\gamma$ and to obtain amounts of $\beta\gamma$ which were as high as that of receptor, we used urea-treated membranes which we have previously shown are a good system for assessing receptor−G protein coupling (*32*). Effects of urea treatment and $\beta\gamma$ reconstitution on F-α_{i1} binding to the CHO membranes are illustrated in Figure 4. There was a small amount of F-α_{i1} binding to non-urea-treated membranes (e.g., Figure 4A, 10 nM F-α_{i1}). After urea extraction specific binding of F-α_{i1} to the membranes was eliminated at low F-α_{i1} concentrations (10 nM or less; see Figure 4C). Reconstitution of $\beta\gamma$ into both control and urea-treated membranes resulted in a dramatic increase of F-α_{i1} specific binding (Figure 4B,D). The urea-treated membranes showed slightly more F-α_{i1} binding observed possibly due to greater exposure of the $\beta\gamma$ or higher efficiency of $\beta\gamma$ incorporation into the urea-treated membranes.

Evidence for functional coupling of the reconstituted $\alpha\beta\gamma$ heterotrimer with expressed α_{2A}AR in urea-treated CHO membranes is shown in Figure 5. Reconstitution of either α or $\beta\gamma$ subunits alone did not promote agonist binding. When both α and $\beta\gamma$ were reconstituted, high-affinity binding of the agonist [^{125}I]-*p*-iodoclonidine (PIC) to α_{2A}AR increased 10-fold. The binding was guanine nucleotide sensitive, indicating functional RG coupling.

With this system established, we asked whether the affinity of F-α_{i1} binding to $\beta\gamma$ was altered in the presence of receptor. We expected that the receptor might provide additional interaction energy to bring the F-α_{i1} more strongly to the membrane. Thus we measured binding of F-α_{i1} to $\beta\gamma$ reconstituted either into control CHO cell membranes or into membranes expressing a high level of α_{2A}AR (Figure 6). Under the conditions of this reconstitution there should be about 1 receptor per 2 $\beta\gamma$ subunits so about half of the $\beta\gamma$ subunits should be able to associate with a receptor. To enhance the contribution of receptor to the binding of F-α_{i1}, we tested the effect of the full α_2 adrenergic agonist, UK-14304, on F-α_{i1} binding to the receptor−$\beta\gamma$ membranes with the idea that a tight DRG ternary complex would increase the contribution of receptor. There was no significant F-α_{i1} binding detected for the urea-treated CHO membranes in the absence of reconstituted $\beta\gamma$ while addition of as little as 0.4 nM $\beta\gamma$ yielded well-defined saturation isotherms (Figure 6A). All equilibrium binding curves overlapped with K_d values ranging from 1.18 to 1.34 nM, determined by nonlinear least-squares regression analysis. The lack of effect on binding affinity was confirmed in Scatchard plots (Figure 6B). Thus, the receptor did not produce any significant enhancement (or reduction) in the binding of F-α_{i1} to the brain $\beta\gamma$ in the membranes.

Since the apparent K_d is similar to the concentration of $\beta\gamma$ present, it is possible that we were just measuring a titration of the $\beta\gamma$ present and that the affinity was so tight

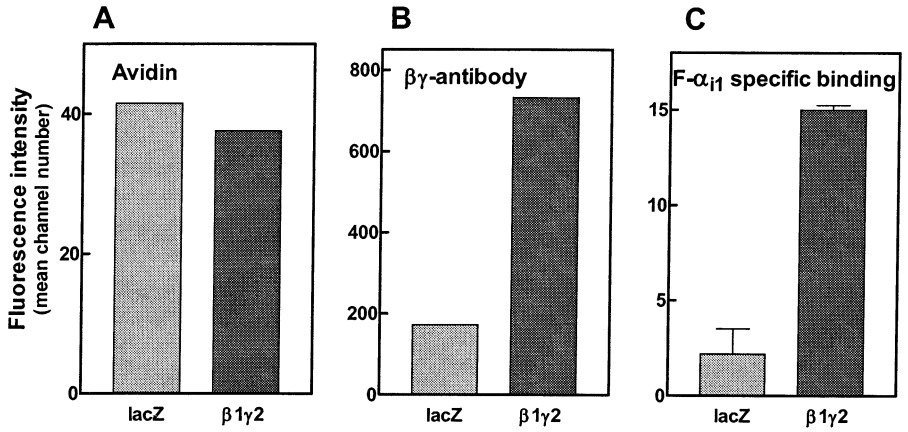

FIGURE 3: Analysis of Sf9 cell membranes by flow cytometry. Control and $\beta\gamma$-expressing Sf9 cells were labeled with biotin−Con-A; membranes were isolated, coupled to the avidin-covered beads, and tested by flow cytometry assay as described in Experimental Procedures. The 10 μg/mL control and $\beta\gamma$-expressing Sf9 cell membranes coupled to the avidin beads were compared for (A) F-avidin binding, (B) detection of $\beta\gamma$ by FITC-labeled secondary and $\beta\gamma$-specific primary antibodies, and (C) specific binding of 10 nM F-α_{i1}. The nonspecific F-α_{i1} signal was defined by binding in the presence of a 50-fold excess of unlabeled α_i and represented less than 10% of the specific binding signal.

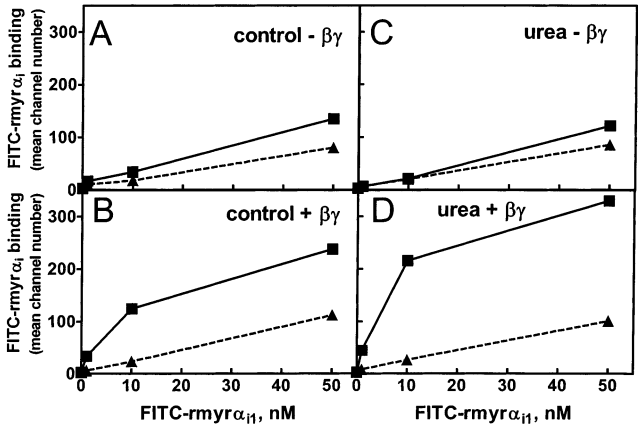

FIGURE 4: Reconstitution of $\alpha-\beta\gamma$ binding in urea-treated CHO cell membranes. CHO cell membranes expressing 45 pmol/mg HA-tagged α_2AR and labeled with biotin−Con-A were compared before and after treatment with 6 M urea. Purified bovine brain $\beta\gamma$ (23 nM final) was reconstituted into both urea-treated and control membranes. Membranes (5 μg/mL) were coupled to the avidin-covered beads, and binding of the indicated concentrations of F-α_{i1} was compared in all four conditions. Total (■) and nonspecific (▲) binding of F-α_i was determined, with nonspecific defined in the presence of a 50-fold excess of r-myr-α_i. Panels: (A) control CHO membranes; (B) control membranes with the $\beta\gamma$ reconstituted; (C) urea-treated membranes; (D) urea-treated membranes with the $\beta\gamma$ reconstituted.

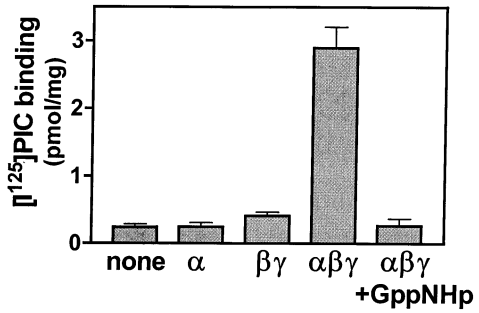

FIGURE 5: Functional coupling of the α_2AR−HA receptor and G protein in urea-stripped CHO cell membranes. CHO cell membranes expressing the α_2AR were urea-washed and treated with biotin−Con-A as described in Experimental Procedures. Membranes (60 fmol of receptor per tube) were reconstituted with buffer alone, a 25-fold molar excess of purified myristoylated α_{i1} alone, an 80-fold molar excess of bovine brain $\beta\gamma$, or both subunits together. Following a 1 h incubation, membranes were diluted 5-fold into binding assay buffer with 1 nM agonist [^{125}I]PIC. With both subunits added, binding was determined in the presence or absence of 10 μM Gpp(NH)p. Data are means ± SE of three experiments performed in triplicate.

that a receptor-mediated increase in binding was overlooked. Also, the receptor is only present at a stoichiometry of 1:2 for $\beta\gamma$ so any effect might have been masked by the excess $\beta\gamma$ which could not interact with receptor. If formation of a ternary complex with receptor did tighten the interaction between G protein subunits, then we would have expected that the rate of F-α_{i1} dissociation from the R−$\beta\gamma$ complex would be reduced. Indeed, dissociation kinetics are a very sensitive method to detect both increased affinity and heterogeneity in binding as might result from the substoichiometric amount of receptor (*38*). Thus, we measured the rate of the F-α_{i1} dissociation from the $\beta\gamma$ in the CHO membranes (Figure 7). One nanomolar F-α_{i1} was brought to equilibrium with 0.4 nM $\beta\gamma$ reconstituted into control membranes and membranes expressing 0.2 nM α_{2A}AR in the presence or absence of UK-14304. Addition of a 50-fold excess of unlabeled α_{i1} induced the rapid exchange of unlabeled Gα subunits for the F-α_{i1} on the $\beta\gamma$−receptor docking site. Loss of fluorescence on the beads was monitored in real time following about a 5 s delay after addition of unlabeled Gα. The dissociation kinetics of F-α_{i1} from all membrane preparations tested were identical with a $t_{1/2}$ of ~3 min at room temperature. There was no evidence for a component of the dissociation that occurred more slowly, which would have been expected if the receptor increased the affinity of F-α_{i1} association to a fraction of the $\beta\gamma$ subunits.

Binding of an agonist initiates a series of the conformational alterations, mainly in receptor and Gα subunit, leading to the exchange of guanine nucleotides. This presumably causes dissociation of G protein heterotrimer from the receptor and in some systems is accompanied by release of α into the cytoplasm (*39*, *40*). We thus examined the Fα−$\beta\gamma$−α_{2A}AR ternary complex in CHO membranes as a model for the real-time analysis of the receptor-induced destabilization of the G protein heterotrimer upon activation and nucleotide exchange. F-α_{i1} was added to membranes contain-

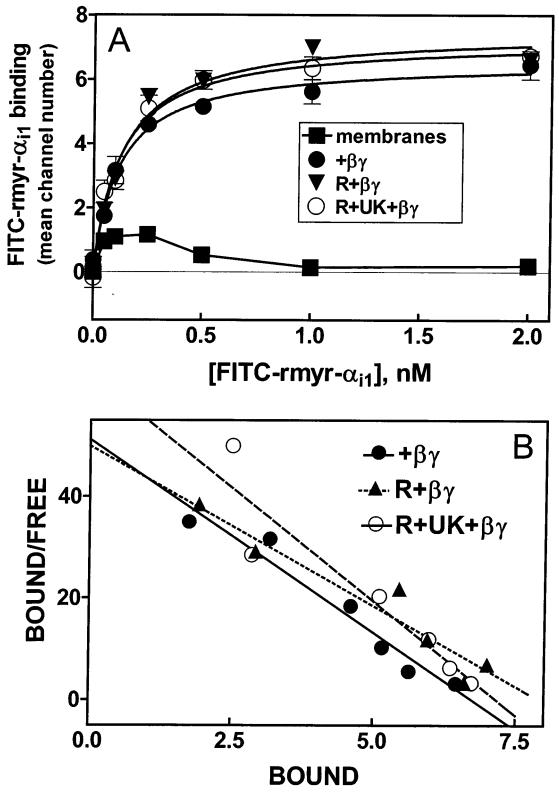

FIGURE 6: Receptor with or without agonist does not enhance the affinity of GDP-bound F-α_{i1} for $\beta\gamma$ in membranes. Plasma membrane fragments of control and α_2AR−HA expressing CHO cells were labeled with biotin−lectin and then treated with 6 M urea. $\beta\gamma$ was reconstituted into both control and α_2AR-expressing CHO membranes. Membranes were bound to the avidin-coated beads in HEDNM(BSA) so that the final concentrations were 0.4 nM $\beta\gamma$ and 0.2 nM receptor in 5 μg/mL membranes on 10^5 beads/mL density of the beads. F-α_{i1} at the indicated concentrations was incubated with the beads for 30 min at room temperature, and fluorescence associated with the beads was measured by flow cytometry. (A) Equilibrium binding of F-α_{i1} to the control CHO membranes (■), $\beta\gamma$-reconstituted membranes (●), $\beta\gamma$ + receptor (▼), or $\beta\gamma$ + receptor + 100 nM UK-14304 (○). Data are means ± SD of two independent experiments. (B) Scatchard plot of equilibrium binding of F-α_{i1} to the various membranes with $\beta\gamma$ present.

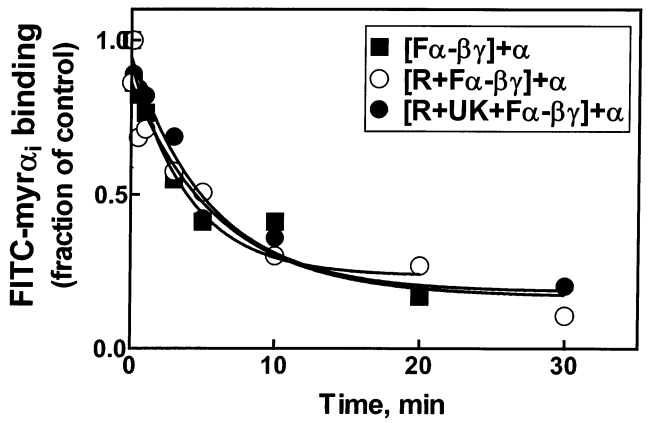

FIGURE 7: Receptor and agonist do not slow the dissociation kinetics of GDP-bound F-α_{i1} from $\beta\gamma$. Control and α_2AR−HA receptor expressing CHO membranes were stripped by 6 M urea treatment in order to remove any endogenous $\beta\gamma$ and then labeled by avidin (see details in Experimental Procedures). Membranes (5 μg/mL) with and without 0.2 nM α_2AR receptor were reconstituted with 0.4 nM purified $\beta\gamma$ and then coupled to the biotin beads. To form the receptor−G protein ternary complex, a portion of the membranes was preincubated with 100 nM UK-14304 for 5 min at room temperature. Then 1 nM of F-α_{i1} was added to all samples, and the mixture was brought to equilibrium by 1 h incubation at room temperature. Dissociation of the F-α_{i1} was initiated by adding a 50-fold excess of unlabeled r-myr-α_{i1}. Data are representative of two independent experiments.

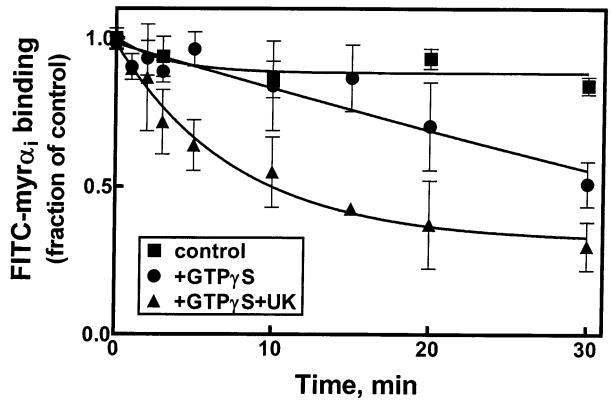

FIGURE 8: Activation of the receptor facilitates the release of Gα from the cell membrane. The purified $\beta\gamma$ was reconstituted into the α_2AR−HA receptor expressing urea-stripped CHO cell membranes. Biotin−lectin-labeled membranes containing 0.2 nM α_2-AR−HA receptor and 0.4 nM $\beta\gamma$ were coupled to the avidin beads in HEDNM(BSA) buffer. FITC−r-myr-α_{i1} (1 nM) was added to the mixture, and the resultant mixture was incubated for 1 h at room temperature. Where indicated, 500 nM GTPγS was added for 5 min. Dissociation of F-α_{i1} was then examined at 30 °C under three conditions: control membranes without additives (■), 500 nM GTPγS (●), and 500 nM GTPγS and 100 nM UK-14304 (▲). Samples were withdrawn at the indicated time intervals from the time point of UK-14304 addition and immediately placed on ice until flow cytometry analysis within 20 min. Data are means ± SE of three independent experiments, each performed in duplicate.

ing 0.4 nM $\beta\gamma$ in CHO membranes with or without 0.2 nM α_{2A}AR preassembled on avidin beads. Subunit dissociation was initiated by addition of a nonhydrolyzable GTP analogue (GTPγS) in the presence and absence of agonist. Preliminary experiments at room temperature showed little release of F-α_{i1} so subsequent experiments were done at 30 °C (Figure 8). In the absence of GTPγS there was almost no dissociation of F-α_{i1} from the membranes (filled squares). Addition of GTPγS caused very slow dissociation of F-α_{i1} with a half-time of greater than 20 min. Preactivation of the receptor with 100 nM UK14304 significantly accelerated GTPγS-induced F-α_{i1} dissociation from the $\beta\gamma-\alpha_{2A}$AR complex ($t_{1/2}$ ~5 min). This stimulated release of F-α_{i1} from $\beta\gamma$ on the membranes demonstrates two things. First, there is significant functional coupling of the α_{2A}AR in these reconstituted membranes so the lack of effect of receptor and agonist on F-α_{i1} binding affinity is not due to a lack of RG coupling. Second, the F-α_{i1} in this study is released from the membrane on a relatively rapid time scale, and the flow cytometry approach permits real-time monitoring of that release. While G_s and G_q α subunits have been shown to be released from cell membranes, this has not been commonly observed for G_i family G proteins (see Discussion).

DISCUSSION

This new biophysical approach permits us to study protein−protein interactions in a lipid environment on the second time scale with picomolar sensitivity. Furthermore, we present novel information about affinity and kinetics of

G protein subunit interactions. Sklar and co-workers pioneered the application of flow cytometry to the study of high-affinity ligand−receptor interactions (*41*) and more recently used the same approach to study protein−DNA interactions (*35*) and protein−protein interactions (*50*). We have extended this to study protein−protein interactions in a lipid environment (*25*). In the present report, we quantitate the contribution of Gα subtype and lipid modification on $\alpha-\beta\gamma$ subunit interactions and examine, in a biological membrane context, the effect of α_{2A}AR on $\alpha_{i1}-\beta\gamma$ interactions.

The range of literature estimates of $\alpha-\beta\gamma$ binding affinities was reviewed in our previous flow cytometry study of $\alpha_{i1}-\beta\gamma$ interactions (*18*), and the present data permit us to systematically compare directly measured $G\alpha-\beta\gamma$ affinities for differentially lipid-modified α_{i1}, α_o, or α_s isoforms in various environments. Our competition binding results in detergent solution agree best with those of Ueda et al. (*42*), who used inhibition of steady-state GTPase as a functional measure of $\alpha-\beta\gamma$ interactions. The 0.2 nM K_d for α_{i1} is almost exactly the same; however, our affinities for r-myr-α_o and non-lipid-modified α_s are about 10-fold lower than they observed. It has been suggested that unknown post-translational modifications of α_s may be present which could enhance its affinity for $\beta\gamma$, but we cannot address that in the present study. In comparison to other direct measures of subunit binding (*43*) our results give K_d values about 10−100-fold lower for both α_{i1} and α_o, probably because the concentration of the $\beta\gamma$ in our flow cytometry competition binding assay was 50 pM, which is low enough to avoid titration phenomena. The physiological significance of the markedly lower $\beta\gamma$ binding affinity of α_s and α_o for $\beta\gamma$ compared to that of α_{i1} remains to be determined.

While myristoylation dramatically increased affinities between α_{i1} and α_o with $\beta\gamma$ subunits in detergent solution, reconstitution of the $\beta\gamma$ into a lipid environment did not produce any further enhancement of the myristate effect on $\alpha-\beta\gamma$ binding. These data are consistent with the myristate having significant contacts with the $\beta\gamma$ subunit proteins or with the prenyl group on γ. Alternatively, any role of lipids in binding myristate is preserved in the nonionic detergent Lubrol. Interestingly, addition of the positively charged His_6 modification on the amino terminus of α_{i1}, which is frequently used in protein purification, partially mimics the hydrophobic enhancement of G protein heterotrimer interaction in detergent solution but does so significantly less well in the lipid environment. This may be due to positively charged lipid headgroups of the phosphatidylcholine limiting association of the His_6 group. Attempts to address the role of palmitoylation (see Results) were not conclusive as no increase in membrane interactions of F-α_{i1} was seen. In light of recent observations that palmitoylation may target proteins to caveolae or lipid rafts (*51*), it is perhaps not surprising that we saw no difference when using a brain phospholipid vesicle which would not have the cholesterol enrichment of that unique environment.

Understanding the dynamics of the receptor and G protein coupling is essential for defining the molecular mechanism of their function. The availability of reconstitution systems has permitted kinetic analysis of functional interactions of the receptor and G protein subunits so that the rate and equilibrium constants of individual activation steps can be defined. In this study, we attempted to move from simplified in vitro systems with purified detergent-solubilized components to a more nativelike membrane environment. Furthermore, we define the physical interaction in real time. In our experiments with the receptor−G protein ternary complex assembled in Sf9 or CHO cell membranes, we focused on two questions: (1) does receptor interaction significantly enhance F-α_{i1} binding to $\beta\gamma$ and (2) how does activation of the receptor modulate F-α_{i1} binding in the ternary complex?

Formation of the membrane-embedded ternary complex transiently latches receptor and $G\alpha\beta\gamma$ subunits together to transduce the activation signal across the plasma membrane. It is has been predicted that G protein binding to the receptor would be enhanced upon agonist binding, and this has been demonstrated in experiments with detergent-solubilized receptor systems (*44*). Several lines of evidence suggest that G proteins can precouple to receptors in membranes even in the absence of agonist (*45*−*48*); however, these conclusions were based on indirect functional studies. Since the assembly of a receptor−G protein ternary complex requires both α and $\beta\gamma$ subunits, tighter binding of F-α_{i1} to $\beta\gamma$ in membranes might be expected if receptor is present and contributes significant interaction energy. Interestingly, expression of the α_2AR receptor did not enhance the affinity of F-α_{i1} for $\beta\gamma$. Neither equilibrium binding nor rates of dissociation were altered in the presence of receptor and/or agonist.

There could be a number of reasons why there is no effect of receptor on F-α_{i1} binding to the membranes. First, changes in F-α_{i1} binding to the membranes due to coupling to the receptor could be masked by the high-affinity $\alpha-\beta\gamma$ interaction (K_d 0.2 nM) that is approaching the detection limit of our measurements, but this does not appear to be the explanation since the dissociation kinetics were not slowed. Also, the enhancement of G protein association with receptor could require the nucleotide-free state of the G protein, and our experiments were done in the presence of GDP to stabilize the $\alpha_{i1}-\beta\gamma$ complex. The nucleotide binding site on Gα assembled in the ternary complex, although emptied by the receptor-accelerated GDP release, could fill in quickly by the excess of the free GDP in buffer solution. In particular, it is puzzling that $\alpha_{i1}-\beta\gamma$ binding is not increased since the agonist-stimulated release of F-α_{i1} from the membranes occurs in the same system. Given that the stimulated GTPγS binding and F-α_{i1} release is kinetic while the enhanced F-α_{i1} binding would have to occur at equilibrium to be observed, perhaps there is a transient increase in F-α_{i1} binding to $\beta\gamma$ and the receptor, but it is reversed by the GDP in the mixture so that at equilibrium the effect is minimal.

An effect of receptor was observed, however, in the kinetics of subunit dissociation after GTPγS was added. Activation of the receptor facilitated release of F-α_{i1} from the cell membrane. The half-time for release (5 min) is slower than the onset of many physiological responses. In this experimental system, we have five times as much F-α_{i1} as receptor and only twice as much $\beta\gamma$. As a GTPγS-occupied F-α_{i1} is released from $\beta\gamma$, a soluble GDP-bound F-α_{i1} subunit from the supernatant could rebind the $\beta\gamma$ and would then need to be stimulated by receptor to be released. Thus several rounds of receptor-mediated G protein activation might be required for the complete release of F-α_{i1} from the membrane. Also, release of subunits from the membrane could be slower than the activation process itself, especially if

activation does not require full dissociation of Gα from $\beta\gamma$ but only an opening of the Gα−$\beta\gamma$ complex to expose the activation surfaces of Gα and $\beta\gamma$. This could permit activation and reassociation on a fast time scale without requiring the Gα and $\beta\gamma$ to diffuse back to one another for reassociation. Thus, the release of F-α_{i1} from the membrane might occur only during some (say 1:10) activation events.

It is interesting that we observe such efficient release of the F-α_{i1} from these cell membranes when this has not been reported in whole cell studies. The situation in whole cells may be different due to palmitoylation of the G-α_{i1} subunit in cells (*49*). Also, the urea stripping of our membranes to obtain a good specific F-α_{i1} binding signal may have removed other proteins that could more strongly localize the α subunits to the membrane. Additional studies with labeled α subunits in whole cells will be required to assess the degree to which α_{i1} subunits are released from plasma membranes. This, however, should be a useful measurement to evaluate the kinetics of G protein activation in this type of biochemical system.

In summary, we have used a novel flow cytometry approach to study, in detergent, lipid vesicles, and cell membranes, the association of G protein α and $\beta\gamma$ subunits and their modulation by lipid modification and receptor activation.

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