Real-time Analysis of G Protein-coupled Receptor Reconstitution in a Solubilized System*

Received for publication, October 23, 2900, and in revised form, March 7, 2001
Published, JBC Papers in Press, April 17, 2001, DOI 10.1074/jbc.M009679200

The abbreviations used are: GPCR, G protein-coupled receptor; G protein, guanine nucleotide-binding regulatory proteins; L, ligand; R, receptor; G, G protein; FPR, N-formyl peptide receptor; FITC, fluorescein 5-isoithiocyanate; GTP-γS, guanosine 5′-O-(thio)triphosphate; Ab, antibody; PIPES, 1,4-piperazinediethanesulfonic acid.

Receptor based signaling mechanisms are the primary source of cellular regulation. The superfamily of G protein-coupled receptors is the largest and most ubiquitous of the receptor mediated processes. We describe here the analysis in real-time of the assembly and disassembly of soluble G protein-coupled receptor-G protein complexes. A fluorometric method was utilized to determine the dissociation of a fluorescent ligand from the receptor solubilized in detergent. The ligand dissociation rate differs between a receptor coupled to a G protein and the receptor alone. By observing the sensitivity of the dissociation of a fluorescent ligand to the presence of guanine nucleotide, we have shown a time- and concentration-dependent reconstitution of the N-formyl peptide receptor with endogenous G proteins. Furthermore, after the clearing of endogenous G proteins, purified Ga subunits premixed with bovine brain Gβγ subunits were also able to reconstitute with the solubilized receptors. The solubilized N-formyl peptide receptor and Ga13 protein interacted with an affinity of ~10−6 M with other α subunits exhibiting lower affinities (Ga13 > Ga12 > Ga11 >> Ga1). The N-formyl peptide receptor-G protein interactions were inhibited by peptides corresponding to the Ga1 C-terminal regions, by Ga1 mAbs, and by a truncated form of arrestin-3. This system should prove useful for the analysis of the specificity of receptor-G protein interactions, as well as for elucidation and characterization of receptor molecular assemblies and signal transduction complexes.

GTP-binding regulatory protein-coupled receptors (GPCR) represent the largest class of cell surface receptors. A broad variety of physiological processes depend on this family of seven transmembrane proteins, making them prime targets for drug discovery (1). Several complementary approaches are being taken in this therapeutic effort. One approach is to define novel therapeutic agents including both agonists and antagonists for specific receptors, as well as molecules that block interactions between receptors and their G protein transduction partners. In addition, the interactions between ligands and receptors are beginning to be thoroughly mapped through studies incorporating receptor mutagenesis as well as analysis of the binding and activity of ligand analogs (2). Comparable efforts are being made to describe the molecular mechanisms that influence the specificity of coupling interactions between GPCR and their cognate heterotrimeric G proteins. Several experimental systems for reconstitution provide alternatives for studying receptors and G proteins, such as adding G protein to stripped membranes, pairing receptors and G proteins in phospholipid vesicles (3), and examining solubilized receptor-G protein interactions using gradient centrifugation (4).

The kinetics of the interactions between ligand (L), receptor (R), and G protein (G) are described by the ternary complex model (5). The formation of LRG is required for the activation of the G protein and typically involves the formation of a high affinity receptor, release of GDP, and activation of the G protein through GTP binding (1). Extended ternary complex models have been developed to describe the transition of receptors from inactive to activated states (6). The assembly kinetics of these systems are not completely understood as the available methods do not provide the time resolution required to evaluate all of the steps in the activation process. Moreover, analysis of receptor-G protein interactions in cells is complicated by the difficulty in elucidating the G protein numbers and concentrations within the microdomains of the receptors and the transient association between receptors and G proteins.

To further the understanding of GPCR-mediated processes, we have utilized the N-formyl peptide receptor (FPR), which couples to a pertussis toxin-sensitive G protein and is expressed predominantly on leukocytes (7). This receptor recognizes the bacterially generated N-formyl peptides that act as potent chemoattractants for human phagocytes. The FPR is one of the better characterized receptors in the chemoattractant/chemokine subclass of GPCR (8). It modulates several cell functions including chemotaxis, superoxide formation, and degranulation, as well as influencing nuclear regulation via activation of MAPK cascades (7). It has been assumed that the FPR binds preferentially to a Ga12 protein as this G i isofrom is highly expressed in neutrophils, while Ga13 is expressed at low levels and there is no expression of Ga11 (9, 10). A recent report using chimeric proteins containing the FPR fused to Ga13, Ga12, or Ga11, expressed in SF9 cells, suggested that the FPR couples to each of the G i isofroms with similar efficiency (11). However,
several aspects of this interaction still remain unresolved.

We have previously described a number of real-time assays of ligand-receptor interactions using flow cytometry and fluorescence that have been primarily directed toward viable cells or detergent-permeabilized cells (12–14). These studies characterized wild type and mutant receptors leading to a model of receptor-G protein coupling for the FPR (13). More recently, we were able to assess the efficiency of solubilization of the FPR using fluorescence methods, and have shown that these receptors were able to reconstitute with G proteins in a non-cellular format (15). In this report we have expanded our investigation of the detergent-solubilized FPR with emphasis on the receptor-G protein interaction. Using reconstitution assays, we demonstrate the ability of the receptor to couple with endogenous G proteins, as well as with exogenous G proteins containing specific Ga subunits. We were able to measure the affinities of the complexes and found that the FPR binds to a G protein heterotrimer containing the Ga subunit with somewhat higher affinity than to heterotrimers containing Ga or Ga proteins. The individual α subunits and the βγ complex alone were unable to induce a change in the dissociation of ligand from the receptor indicating a necessity for the G protein heterotrimer.

We were able to inhibit the G protein-receptor interaction with peptides derived from the Ga subunits as well as with anti-Ga antibodies. Finally, we have expanded the system to include analysis of GPCR-arrestin interactions. The methods described here provide an approach to study the mechanism of GPCR interactions with G proteins, arrestins, and other potential targets and at the same time provide a platform for identifying and characterizing novel therapeutic agents.

**Experimental Procedures**

**Reagents and Cell Culture**—The generation of U937 cells transfected with the FPR was previously described (16). Plasticware was obtained from VWR Scientific Co. Chemicals and reagents were obtained from Sigma except where otherwise noted. Cells were grown in tissue culture-treated flasks (Corning) in RPMI 1640 (Hyclone) containing 10% fetal bovine serum (HyClone), 2% L-glutamine, 10 mM HEPES, with 10 units/ml penicillin and 10 μg/ml streptomycin. Cultures were grown in standard tissue culture incubators at 37 °C with 5% CO2, and pas

105 cells/ml. Purified G protein peptides composed of the last 10 amino acids of Gaα, β3, or Gaα, and Gaα, and functional, myristoylated, rat recombiant) were purchased from Calbiochem. The bovine brain βγ complex was isolated and purified as previously reported (17). Arrestins were expressed in Escherichia coli (strain BL21) and purified by sequential heparin-Sepharose and Q-Sepharose chromatography essentially as described (18). Reagents for inhibition of reconstitution include three G protein α subunit blocking peptides composed of the last 10 amino acids of Gaα, Gaα, and Gaα, and anti-Gα antibodies recognizing the C terminus of Gaα, Gaα, and Gaα, and an internal Gaα antibody (Calbiochem).

**Membrane Preparation by N2 Cavitation**—U937 FPR cells were harvested, centrifuged at 200 × g for 5 min, and resuspended in cavitation buffer (10 mM Pipes, 100 mM KCl, 3 mM NaCl, 3 mM MgCl2, 600 μM ATP) at a density of 106 cells/ml at 4 °C. The cell suspension was placed in a nitrogen bomb and pressurized to 450 psi using N2 gas for 20 min at 4 °C. Nuclei and cytoplasmatic material were separated by centrifugation at 1000 × g for 5 min at 4 °C. The supernatant, containing the membranes, was washed 2 times by centrifugation at 135,000 × g for 30 min at 4 °C, then resuspended in HEPES sucrose buffer (200 mM sucrose, 25 mM HEPES, pH 7.1), aliquoted, and stored until use at −20 °C.

**Solubilization of the FPR**—Membranes were thawed and diluted to 1–2 × 106 membrane cell equivalents/ml (CEQ/ml) in binding buffer (30 mM HEPES, 100 mM KCl, 20 mM NaCl, 1 mM EDTA, 0.1% (w/v) bovine serum albumin, 0.5 mM MgCl2). Preparations were maintained at 4 °C throughout the extraction process. Membranes were centrifuged at 135,000 × g for 30 min and resuspended in 150 μl of binding buffer containing protease inhibitor mixture I and 1% n-dodecyl β-maltoside (Calbiochem). Preparations were incubated 60 min at 4 °C with agita
tion. The insoluble fraction was separated by centrifugation at 70,000 × g for 5 min in a Beckman Airfuge. The supernatant, containing the solubilized fraction, was removed and this extract was used for experimentations within 6 h.

**Velocity Sedimentation**—Membranes (1 × 106 cell equivalents) were solubilized and applied to 1 ml of a 5–20% linear sucrose gradient prepared in binding buffer plus 1% n-dodecyl β-maltoside. Gradients were centrifuged at 40,000 rpm in a TLS-55 rotor (Beckman) for 14 h and fractionated into 20 × 50-μl fractions. To establish the distribution of membrane-associated protein, 25 μl of each fraction was incubated with 10 μM fluoresein-3-Met-Leu-Phe-Lys-fluorescein 5-isothiocyanate (FMFF, FITC, Perkin-Elmer) for 2 h on ice. Fractions were subjected to spectrofluorometric analysis, as outlined below. Gradients containing 5 μg of bovine serum albumin (4.4 S) and rabbit immunoglobulin (7.7 S) were centrifuged in parallel. These fractions were analyzed by SDS-polyacrylamide gel electrophoresis, followed by Coomassie staining. Based on the sedimentation of these standard proteins, the migration of 4 S and 7 S proteins was calculated to peak at fractions 9 and 15, respectively.

**Reconstitution of Receptors with G Proteins**—Purified individual α subunits were mixed in an equimolar ratio with the βγ complex and incubated on ice for 15 min to form the heterotrimeric complex (17). Solubilized FPR (5–10 μM) in 1% n-dodecyl β-maltoside prepared as above), was then incubated with the specific G protein heterotrimers or with a mixture of bovine brain Gi2, Go1, and Gi3, and Go3, which contained 1 μM formyl-Met-Leu-Phe-Phe (FMFF, CBI) for 15 min prior to the addition of FPR. Samples were analyzed by FMFF-FITC fluorescence on the spectrofluorometer (19, 20). The derived value for the Gaα subunit concentration was consistent with the value reported by the supplier (data not shown).

**Spectrofluorometric Analysis**—Fluorescence associated with FMFF-FITC was measured by a SLM 8000 spectrofluorometer (SLM Instruments, Inc.) using the photon counting mode in acquisition. The sample holder was fitted with a cylindrical cuvette adapter to permit measurement of the spectral profile of the 515-nm emission (Sinceluy 200 μm slits) and 5-nm slit bars (Sintec) and 2 × 5-nm stir bars (Bel-Art). Excitation was fixed at 490 nm, and stray light was reduced with a 490 nm, 10-nm band pass filter (Corin). FITC fluorescence emission was monitored using a 520 nm, 10-nm band pass interference filter (Corin) and a 3–70 orange glass, 500-nm long pass filter (Kopp). Additions to samples during kinetic measurements were performed using 10-μl glass syringes (Hamilton) adding reagents through an injection port on the SLM 8000 above the sample cuvette.

Following preparation at 4 °C, samples were brought up to a volume of 200 μl with binding buffer plus 0.1% n-dodecyl β-maltoside, equilibrated to 22 °C, and placed into the spectrofluorometer with constant stirring. Data were acquired for 180–210 s in 1-s intervals. Typically, total fluorescence was obtained for the first 20 s, then, an anti-fluoresscin antibody was added to the sample. The antibody binds free FMFF-FITC with high affinity and results in essentially complete quenching of the fluorescence associated with free ligand (14). Thus, the remaining fluorescence represents the receptor-bound. In G protein experiments, GTPγS (100 μM) sensitivity was used to assess the coupling between receptors and G proteins based on characteristic ligand dissociation rates. Experiments were performed using a detergent concentration slightly above the critical micelle concentration.

**Statistical Analysis**—Data were analyzed and graphed using Prism software (Graph Pad Software Inc.). To determine the dissociation characteristics of the receptor preparations, the fluorescence over time in blocked control samples was subtracted point by point from the fluorescence over time of ligand binding samples as described previously (14). The data were then normalized and the relative fluorescence was fit by nonlinear regression using single (Equation 1) or double (Equation 2) exponential decay equations.

\[
I = A_1 e^{-kt_1} + \text{plateau}
\]  
(1)

\[
I = A_1 e^{-kt_1} + A_2 e^{-kt_2} + \text{plateau}
\]  
(2)
Where $I = \text{fluorescence intensity in arbitrary units, } k, t = \text{the fluorescence when all peptide has dissociated and } A_t = \text{the fraction of receptor in the state with the rate of } k$. Rates are given as mean ± S.E.

**Clearing Sample of Endogenous G Protein—**Membrane preparations were solubilized in detergent as outlined above. The sample was then incubated with 20 μl of anti-Gα1,2,3 antibody (Calbiochem) for 45 min on ice. To remove antibody-substrate conjugates, 100 μg of Protein A-agarose was added to the sample. After a 30-min incubation, the sample was centrifuged at 14,000 $\times g$ for 30 s and the supernatant was removed. The process was then repeated.

**Western Blot Analysis—**Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Gelman) with a semi-dry transfer apparatus (Owl Scientific). Membranes were blotted with antibody (rabbit) against either Gα1,2,3 or βγ (Calbiochem) followed by an horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Sigma). The blots were developed using ECL Plus (Amsersham Pharmacia Biotech) and imaged using a PhosphorImager (Molecular Dynamics).

**RESULTS**

**Fluorometric Assay Development—**Receptor-G protein interactions have been characterized in intact and permeabilized cells, membranes, phospholipid vesicles, and in detergent using gradients (3, 21–23). We have extended the use of detergent-solubilized FPR and G proteins, adding fluorescence detection, to study receptor-G protein complexes in real-time. We have taken advantage of the fact that the ligand dissociation rate of the LR complex of the FPR (in the absence of G protein or in the presence of GTPγS) is many times faster than ligand dissociation from LRG (12, 24, 25). Thus, the presence of the G protein imparts a higher affinity for ligand to the receptor. When GTP is not available, the G protein remains bound to the FPR. The addition of GTP, or its non-hydrolyzable analog GTPγS, putatively induces the dissociation of G protein from the receptor and results in a decrease in the affinity of the ligand, increasing its dissociation rate. The change in the ligand dissociation rate is likely to represent a switch from the slowly dissociating LRG to the rapidly dissociating LR as the activated G protein uncouples from the FPR.

The assay is based upon a fluorescein-conjugated ligand used along with an anti-fluorescein antibody that rapidly quenches the fluorescein of the free ligand upon binding. The antibody is able to interact only with the fluorescein on the free ligand, as FPR-ligand complexes sterically inhibit the antibody from binding the fluorescein (14). In this way we were able to determine the quantity of FPR-bound ligand (Fig. 1) immediately following the addition of the anti-fluorescein antibody. We were also able to follow the ligand dissociation kinetics as the excess antibody further quenches ligand released from the receptor. These methods were based on assays that have been fully characterized using permeabilized whole cells (12–14). The dissociation data was analyzed as described under “Experimental Procedures.” The data were fit to one or two rates, the slow rate apparently representing L dissociation from RG and the fast rate apparently representing L dissociation from R.

**Endogenous G Protein Reconstitution with a Solubilized Receptor—**The human FPR was previously stably transfected into the undifferentiated human myeloid cell-line, U937 (16). The U937 cells expressing the wild type FPR react specifically to formylated peptides with calcium and chemotactic responses (26). Membrane fractions were prepared using N2 cavitation and the work presented is data from both intact membranes or membranes that have been solubilized with 1.0% detergent solution. The extent of ligand binding to the FPR is similar for membranes and the solubilized membrane extract (15). However, in the membrane samples it appeared that G proteins were either pre-coupled to, or interacted almost immediately with, the receptor after the addition of ligand, but once the membranes were solubilized, no G protein interaction was observed over the same time frame (Fig. 1A). In earlier studies we demonstrated the ability of the solubilized receptor to interact with an exogenous G protein, if the receptor was incubated in the presence of ligand for 2 h (15). Thus it was possible that the solubilized receptor would reconstitute with endogenous G proteins over time if ligand was present. As shown in Fig. 1B, there was indeed a time dependence to the interaction. Additionally, when assays were prepared in larger volumes, that diluted the concentration of both receptor and G protein, reconstitution was not observed (data not shown). This dependence on receptor and G protein concentration in solution suggests that while within the membranes, the receptor-G protein interactions take place rapidly in two dimensions, the solubilized proteins exist in three dimensions requiring extra time for the proteins to associate.

To confirm that our receptor preparations were solubilized in the presence of 1% detergent (n-dodecyl β-maltoside), a sucrose velocity sedimentation assay was performed. This assay determines the extent of solubilization and complex formation of proteins. Briefly, the solubilized receptor fraction is layered onto a 5–20% sucrose gradient. Following centrifugation, the gradients are fractionated, and the position of the FPR within the gradient was determined by spectrofluorometric analysis.

---

**FIG. 1. Receptor-G protein coupling in membranes and solubilized receptor.** A, a membrane sample from U937 cells stably transfected to express the FPR was washed once, resuspended into 150 μl of binding buffer, then divided into two aliquots. One of these was solubilized by incubating for 1 h at 4°C in the presence of 1.0% dodecyl maltoside then centrifuged to remove the insoluble fraction. The supernatant was collected, containing the solubilized fraction. To compare ligand binding and dissociation of the membrane preparation versus the solubilized sample, 10 μl of each was added to a glass cuvette along with 1 μM ligand (fMLFK-FITC) in a final volume of 200 μl and equilibrated to room temperature for 2 min with stirring. Samples were placed in the fluorometer, at 20 s an anti-FITC-Ab was added and then at 120 s GTPγS was added. Graph displays plot of fluorometric analysis of the dissociation of the fluorescent ligand. B, approximately 10 nm solubilized receptor (10 μl) was incubated at 4°C with indicated amounts of FPR for the indicated times. Prior to analysis, samples were equilibrated to room temperature with constant stirring. The anti-FITC-Ab was added at 20 s and GTPγS at 120 s. Plots are representative of three experiments each done in duplicate.
The FPR was found in fractions 8–10, sedimenting as a 4 S species, as expected for a monodisperse receptor (Fig. 2). Also, this further confirmed that the solubilized FPR was not initially coupled to a G protein.

Clearance of Endogenous G Proteins from Solubilized Membrane Preparations—In order to generate a system in which the affinities of specific exogenous G protein subunits could be determined for the FPR, we first needed to remove the endogenous G protein from our solubilized receptor solution. This was accomplished by incubating the sample with an anti-Ga1,2,3 antibody then adding protein A-agarose to clear the antibody-substrate conjugates. Our solubilized receptor sample, when treated in this manner, lost all sensitivity to GTPγS and the non-linear regression fit of the data indicated only a single exponential decay corresponding to LR. This suggested that the endogenous G proteins had indeed been removed from the sample (Fig. 3A). Western blot analysis of the sample prior to, and after antibody treatment, confirmed that this procedure effectively cleared the endogenous G proteins from the system. It appeared that the G proteins were present in the heterotrimeric form as the anti-Ga antibody pulled down both the α subunits and the βγ complex (Fig. 3B). A receptor preparation treated in this way would be appropriate for the analysis of specific receptor-G protein interactions.

Reconstitution of the Solubilized FPR with G Proteins Containing Specific Ga Subunits—It has been shown that the FPR interacts with pertussis toxin-sensitive Gα proteins in neutrophils and U937 cells (9, 10). The predominantly expressed Gα protein in neutrophils has a Ga12 subunit. Thus it has been assumed that the FPR preferentially binds to the Ga12 proteins. Until now it has been difficult to directly assess the affinity between G proteins and receptors. We have previously demonstrated the ability of solubilized receptors to reconstitute with a bovine brain G protein mixture with an ED_{50} of ~10^{-6} M under conditions where the ligand and receptor concentrations are ~10 nM (15). Expanding this study, we examined this interaction using specific, purified G protein α subunits (Ga11, Ga12, Ga13, and Ga3). The individual α subunits were first mixed in an equal molar ratio with bovine brain βγ to form a G protein heterotrimeric complex (17). Solubilized receptor, that had been depleted of endogenous G protein, was incubated with the individually prepared G protein heterotrimers in the presence of fluorescein-conjugated ligand for 2 h. The resulting plots, as seen in Fig. 4, A–D, demonstrate the preference of the FPR for the Ga11 subunits as the Ga3 protein does not appear to induce the high ligand affinity state of the receptor. Non-linear regression of the first phase (t = 20–90 s) of the Ga1 curves (*) fit to a double exponential (after subtraction of the free component), indicating that two different rates were present. The slower rate was 0.0065 ± 0.0020 s^{-1}, representing ligand dissociation from RG (high affinity state), and the second rate was 0.0309 ± 0.0069 s^{-1}, ligand dissociation from R (low affinity state). The amplitudes were used to determine the percentage of the receptors in the sample that were initially coupled to the G protein (Fig. 5). The data indicates that the FPR interacts with the Ga11-specific G protein with an apparent KD of 1 μM with the other α subunits displaying lower affinities (Kd Ga12 ≈ 1 μM < Kd Ga13 < Kd Ga31 ≈ Kd Ga32 ≈ Kd Ga33). As G protein concentrations of 3 μM were the highest we could obtain, the Kd for the other G protein subunits could only be estimated. The Ga11, and Ga12, G proteins have a Kd greater or equal to 3 μM and the Ga3 G protein Kd is likely to be greater than 10 μM given the levels of receptor-G protein coupling observed. The Ga3 data as well as the lower concentrations of Ga11 and Ga12 (t = 20–120 s) was best fit to a single exponential equation with a rate of 0.0315 ± 0.0052 s^{-1}. The ligand dissociation after the addition of GTPγS in all the curves (t = 51–120 s) was also fit by a single exponential decay equation, exhibiting a rate of 0.0325 ± 0.0063 s^{-1}. These rates represent the mean ± S.E. of the values obtained in Fig. 4. There was not a systematic difference between the rates of ligand dissociation from LRG or from LR for any of the Ga subunits as represented in Table I.

Requirement for the G Protein Heterotrimer to Induce Binding to the Receptor—In the previous set of experiments we combined the G protein α subunit and the βγ complex prior to mixing with receptor. Mixing the subunits in this way results in them combining to form an αβγ heterotrimer (17). After reconstituting receptor-G protein interactions with these heterotrimeric G proteins we next investigated whether the α subunits or the βγ complex alone could generate a high affinity state in the FPR. The solubilized receptor was incubated for 2 h.
with either a specific α subunit alone or bovine brain βγ alone. As it appeared that G proteins containing the G\(i\) subunit bound to the receptor with higher affinity, the G\(i\) subunit was tried first. The spectrophotometric analysis showed that neither the G\(i\) subunit nor the βγ complex on their own were able to induce the slow ligand dissociation rate or any sensitivity to guanine nucleotide (Fig. 6A and B). However, if the subunits are combined as described above, receptor-G protein coupling was restored (Fig. 6C). This suggests that the G protein heterotrimer is necessary for generating the high affinity state of the receptor.

**Inhibition of the FPR-G Protein Interaction**—The Gα surface that interacts with the receptor has been well defined in several systems especially for the visual GPCR rhodopsin and its interaction with transducin (27). As these studies implicated the C-terminal region of the subunit as important in binding to the receptor, we next tested the ability of a series of anti-Gα antibodies to interfere with endogenous G protein coupling to the FPR. The antibodies were added to the reconstitution assays at the start of the 2-h incubation. Two anti-Gα antibodies, anti-G\(a_{1,2}\), and anti-G\(a_{3}\) each recognizing the C terminus of the α subunits, was able to inhibit the receptor-G protein interaction (Fig. 7). Conversely, an anti-G\(a_{1}\) C-terminal antibody had no effect and an anti-G\(a_{3}\) antibody, that recognized an internal sequence, likewise had no effect (data not shown).

Further investigation of the receptor-G protein interface was accomplished by incorporating Gα C-terminal peptides into the assays. Three peptides were tested, the first was a G\(a_{1}\), C-terminal peptide (the last 10 C-terminal amino acids of G\(a_{1}\)) (Fig. 8). In both cases the slow ligand dissociation and guanine nucleotide sensitivity was inhibited in the presence of the peptide. While both peptides were effective in disrupting the association of the receptor with the G protein, the G\(a_{1}\) peptide EC\(50\) was \(-0.1\) mM compared with an EC\(50\) of 1 mM for the G\(a_{1,2}\) peptide. The Gα peptide had no effect on the ligand dissociation kinetics and did not interfere with the guanine nucleotide sensitivity (data not shown).

The same experiments were also performed using membrane preparations in place of solubilized receptors. Interestingly, none of the peptides or the antibodies was able to inhibit the FPR-G protein interaction under the same conditions (data not shown).

**FPR-Arrestin Interactions**—The desensitization of activated GPCR by arrestin molecules has been well documented (28). It has been presumed that the arrestin-receptor interaction physically blocks the inactivated G proteins from associating with the receptor (29). The data obtained in the above series of experiments demonstrated that our fluorometric assay was able to quantitate G protein coupled versus uncoupled receptor. This suggested that our methods would be amenable to a receptor-arrestin study. The FPR colocalizes with arrestin-2 and arrestin-3 in U937 cells transfected to express the FPR and in neutrophils (30). The addition of wild-type arrestin-3 had no effect on the ligand dissociation rate or sensitivity to guanine nucleotide (Fig. 9). This was expected as desensitization of GPCR by arrestin is dependent on the phosphorylation of the activated receptor. The use of solubilized receptors in our sys-

---

2 T. A. Bennett and E. R. Prossnitz, unpublished observation.
The relative fluorescence was fit by nonlinear regression using single or double exponential decay equations. Curves displaying sensitivity to GTP·S also fit to a single exponential decay ($t = 20–120$ s). Rates are given as mean ± S.E. in s$^{-1}$.

<table>
<thead>
<tr>
<th>Subunit</th>
<th>$t = 20–50$ s</th>
<th>$t = 51–120$ s</th>
<th>$t = 20–120$ s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ga$_{i3}$-3.0 μM</td>
<td>$k_1 = 0.0078 ± 0.0015$</td>
<td>$k = 0.0317 ± 0.0065$</td>
<td>$k = 0.0345 ± 0.0063$</td>
</tr>
<tr>
<td>1.0 μM</td>
<td>$k_1 = 0.0057 ± 0.0020$</td>
<td>$k = 0.0290 ± 0.0070$</td>
<td>$k = 0.0342 ± 0.0053$</td>
</tr>
<tr>
<td>0.3 μM</td>
<td>$k_1 = 0.0057 ± 0.0018$</td>
<td>$k = 0.0277 ± 0.0063$</td>
<td>$k = 0.0348 ± 0.0063$</td>
</tr>
<tr>
<td>Ga$_{i3}$-3.0 μM</td>
<td>$k_1 = 0.0058 ± 0.0021$</td>
<td>$k = 0.0277 ± 0.0063$</td>
<td>$k = 0.0286 ± 0.0073$</td>
</tr>
<tr>
<td>1.0 μM</td>
<td>$k_1 = 0.0030 ± 0.0059$</td>
<td>$k = 0.0277 ± 0.0063$</td>
<td>$k = 0.0307 ± 0.0060$</td>
</tr>
<tr>
<td>0.3 μM</td>
<td>$k_1 = 0.0072 ± 0.0025$</td>
<td>$k = 0.0300 ± 0.0068$</td>
<td>$k = 0.0339 ± 0.0062$</td>
</tr>
<tr>
<td>1.0 μM</td>
<td>$k = 0.0317 ± 0.0035$</td>
<td>$k = 0.0325 ± 0.0062$</td>
<td>$k = 0.0325 ± 0.0038$</td>
</tr>
<tr>
<td>0.3 μM</td>
<td>$k = 0.0302 ± 0.0055$</td>
<td>$k = 0.0332 ± 0.0043$</td>
<td>$k = 0.0325 ± 0.0038$</td>
</tr>
</tbody>
</table>

DISCUSSION

We have presented here a series of reconstitution assays that have analyzed the ability of the FPR to interact with several G proteins and arrestins. The reconstitution assays depended on the detergent micelles supporting the structural conformation of the FPR adequately for it to retain its ability to bind both ligand and the various proteins that interact with the intracellular domain of the receptor. It was demonstrated that the solubilized FPR was present as a monodisperse, uncoupled protein (Fig. 2). This work has led to the development of fluorometric methods that can systematically evaluate GPCR interactions in real-time. As this was done in a non-cellular format, a controlled environment could be maintained in which specific interactions were studied.

Fluorometric methods that analyze solubilized receptors by real-time ligand binding were utilized. The kinetic data of ligand dissociation was statistically analyzed to determine the off-rate. Bound ligand fluorescence was fit by non-linear regression using single or double exponential decay equations. Receptors that did not associate with G proteins fit best to a single exponential decay. Conversely, when ligand dissociation displayed an initial slow rate, the curve was best fit to a double exponential decay equation, indicating that some fraction of the receptors were bound to a G protein. Moreover, when GTP·S was added to these samples, a rapid dissociation rate was induced that fit to a fast single exponential decay rate (the same rate as $k_1$ and $k$ in Table I) indicating the conversion of receptors in the RG state to R. This allowed for a quantitative measure of the ability of the solubilized receptors to interact with G proteins.

When the FPR was examined in membrane preparations, the ligand-stimulated receptor rapidly formed a G protein-receptor complex, leading to speculation of a receptor-G protein pre-
coupled state. However, with the solubilized receptor, this interaction was highly time- and concentration-dependent (Fig. 1). If precoupling was present in the membrane assays, it is possible the solubilization disrupted the interaction. Additionally, even without precoupling, within the membrane environment the interactions occur over two dimensions, possibly in microdomains. This is in contrast to the three dimensions available in the solubilized sample where LRG assembly is expected to depend on time and [R][G]. Thus in a previous study performed with a lower concentration of solubilized membrane than used here, FPR reconstitution with endogenous G protein was not observed (15). It was fortuitous that the receptor was not precoupled to a G protein in the solubilized system as this allowed for the depletion of the endogenous G proteins (Fig. 3). By isolating all the components of our system, we were able to add specificity to the assembly process.

The next step was to examine FPR interactions with several purified G protein subunits. The G protein containing the Gαi3 subunit had the highest affinity for the solubilized receptor with a Kd of 1 μM (Fig. 4). Obtaining an accurate Kd for the Gαi1 and Gαi2 subunit G proteins was limited by the quantity of available G proteins. However, given the level of receptor-G protein coupling at the 3 μM concentration of G protein, the Kd for Gαi2 would be approximately equal to 3 μM and the Kd for Gαi1 would be greater than 3 μM (Fig. 5). We had previously reported that the solubilized FPR had an apparent affinity for bovine brain G protein, which is predominantly Gαo, similar to what we report here for Gαi3 (15). In this prior work, we had not taken into account the significant contribution endogenous G proteins were having on the system. Consequently, the bovine brain G proteins were in addition to the endogenous G proteins, resulting in a higher total G protein concentration than was used to compute the Kd. With this knowledge, the current work was performed with samples in which the endogenous G proteins were cleared prior to reconstitution with specific subunits.

The addition of individual α subunits or the βγ complex, to samples precleared of endogenous G proteins, did not induce the high affinity state indicative of G protein binding (Fig. 6). While it is not known if these subunits were able to bind to the
receptor or not, they could not induce the resultant state change observed with the addition of the heterotrimer. This also confirmed that the precleared samples were adequately depleted of both α and βγ subunits as the exogenous addition of either component could not induce the high affinity state of the FPR. Inhibition of the receptor-G protein interaction was accomplished through the use of both anti-Gαi antibodies and by Gαq C-terminal peptides. Only antibodies to the Gαq subunits were effective in disrupting the receptor-G protein interaction. However, the anti-Gαq1,2 and the anti-Gαq3 antibodies acted equally (Fig. 7). The similarity of the two Gαq antibody preparations could result from the relatively common peptide sequences which serve as antigens, even though the antibodies have been reported as non-cross-reactive in Western blots. The antibody behavior was in contrast to the results obtained with the Gαq peptides. Concentration curves demonstrated that the Gαq1 peptide EC50 for inhibition was about 10-fold lower than that of the Gαq1,2 peptide (Fig. 8). This correlated well with our previous results showing that G proteins containing the Gαq3 subunit bound to the FPR with higher affinity. The ability of antibodies and especially small peptides to specifically inhibit the receptor-G protein interaction could prove useful in the development of drugs to modify cellular responses by interfering with G protein activation.

This system does not permit definitive conclusions about which G proteins are coupled to receptors under physiological conditions. Considering the FPR, most cells expressing this receptor contain relatively high levels of Gα12 and only small amounts of Gα13 (9, 10). Physiologically, it appears as though the FPR may transduce most of its signal through Gα12 activation even though it may bind preferentially to Gα13. There may be other determinants that operate at the receptor-G protein interface that alter signaling specificity or efficiency. Cell architecture or accessory proteins may differ between cell types that influence potential interactions. A previous report analyzed FPR interactions with the three Gα isoforms and found that the receptor coupled with each with similar efficiency (11). The assay fused the FPR to Gα1, Gα12, or Gα13 and expressed the fusion proteins in Sf9 cells. The use of the fusion proteins creates a high G protein concentration within the microdomain of the receptor, making it difficult to discern difference in binding affinities that are evidenced in the detergent soluble complexes.

The ability to detect the interaction of truncated arrestin-3 with the receptor indicates the potential generality of the assay for the study of many types of molecular assemblies (Fig. 9). Moreover, the effect of arrestin on ligand affinities can be determined. This approach could also be extended to multistep interactions, such as GPCR desensitization, that requires the interaction of the receptor with kinases, arrestins, and cytoskeletal components (28, 32). Another opportunity may be the evaluation of the assemblies of signaling and scaffolding components amenable to the suspension analysis suggested here or particle-based analysis by flow cytometry as suggested previously (15).

REFERENCES


Real-time Analysis of G Protein-coupled Receptor Reconstitution in a Solubilized System
Teresa A. Bennett, T. Alexander Key, Vsevolod V. Gurevich, Richard Neubig, Eric R. Prossnitz and Larry A. Sklar

doi: 10.1074/jbc.M009679200 originally published online April 17, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M009679200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 17 of which can be accessed free at
http://www.jbc.org/content/276/25/22453.full.html#ref-list-1