Independent and synergistic interaction of retinal G-protein subunits with bovine rhodopsin measured by surface plasmon resonance

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We have used surface plasmon resonance (SPR) measurements for the kinetic analysis of G-protein-receptor interaction monitored in real time. Functionally active rhodopsin was immobilized on an SPR surface, with full retention of biochemical specific activity for catalysis of nucleotide exchange on the retinal G-protein α subunit, via binding to immobilized concanavalin A. The binding interactions of bovine retinal α_t and $\beta_1\gamma_1$ subunits with rhodopsin measured by SPR were profoundly synergistic. Synergistic binding of the retinal G-protein subunits to rhodopsin was not observed for guanosine 5′-[γ-thio]triphosphate-bound Gα_t, nor was binding observed with squid retinal Gα_q, which is not activated by bovine rhodopsin. The binding affinity (336 ± 171 nM; mean value $\pm$ S.D.) of retinal $\beta\gamma$ for rhodopsin in the presence of retinal α subunit measured by SPR confirmed the apparent affinity of 254 nM determined previously by nucleotide exchange assays. Binding of $\beta_1\gamma_1$, $\beta_1\gamma_2$, and $\beta_1\gamma_{8\text{-olf}}$ dimers to rhodopsin, independently of the α subunit, was readily observable by SPR. Further, these dimers, differing only in their γ subunit compositions, displayed markedly distinct binding affinities and kinetics. The $\beta_1\gamma_2$ dimer bound with a kinetically determined K_d of 13 ± 3 nM, a value nearly identical with the biochemically determined $K_{1/2}$ of 10 nM. The physiologically appropriate $\beta_1\gamma_1$ displayed rapid association and dissociation kinetics, whereas the other $\beta_1\gamma$ dimers dissociated at a rate less than 1/100 as fast. Thus rhodopsin interaction with its native signalling partners is both rapid and transient, whereas the interaction of rhodopsin with heterologous G$\beta\gamma$ dimers is markedly prolonged. These results suggest that the duration of a G-protein-coupled receptor signalling event is an intrinsic property of the G-protein coupling partners; in particular, the $\beta\gamma$ dimer.

Key words: affinity, synergy, transducin.

INTRODUCTION

The heterotrimeric G-proteins mediate a wide variety of cellular signalling pathways initiated by selective interactions with members of the superfamily of rhodopsin-homologous receptors. G-protein signalling selectivity is enhanced by the unique combination of the protein products of three multigene families encoding α, β and γ subunit chains. Whereas the GTP-binding α-subunit chains have been taken to characterize the function of the G-proteins [1], the $\beta\gamma$ subunit dimer has been recognized for some time to contribute importantly to G-protein signalling (reviewed in [2]). Initial work on reconstituting signal transduction in the visual system found that activation of the α subunit of the retinal G-protein transducin by rhodopsin required the presence of the $\beta\gamma$ dimer [3,4]. Those findings have been reiterated with the resolution and reconstitution of β-adrenergic and muscarinic acetylcholine receptors with G-proteins [5–8]. These results therefore indicate an essential role for the $\beta\gamma$ dimer in presenting the α subunit for receptor interaction.

In addition to the primary structural differences between the G-proteins, the γ subunit chain of the $\beta\gamma$ dimer is modified by a cysteyl ether-linked isoprenoid [either C_{15} (farnesyl) or C_{20} (geranylgeranyl)] at the C-terminus, which is characteristic of a plasma-membrane-localized signalling molecule. The isoprenoid modification is believed to be necessary for membrane anchoring of the trimeric $\alpha\beta\gamma$ structure [9]. Membrane anchoring is thought to be essential therefore for the presentation of the α subunits for specific receptor contact; so far, all identified protein structures for receptor selectivity with G-protein have been mapped to the α-subunit chains. However, for Gα subunits modified by palmitic acid, the interaction with the $\beta\gamma$ dimer might not be required for membrane association [10]. The identification of at least five genes encoding β-subunit structures and approx. 12 or more γ-subunit gene products (reviewed in [11]) suggests a less limited role for the $\beta\gamma$ dimer than simply acting as a membrane anchor for the G-protein trimer. Alternative biochemical functions such as the regulation of effector pathways have been described for the G$\beta\gamma$ dimer [2]. Dimers of defined β and γ composition from genes expressed in Sf9 cells have been tested in effector assays for the regulation of adenylate cyclase and phospholipase C-β. Other than retinal γ_1, no impressive distinction arose between the various β and γ chains examined [12,13]. We speculated some years ago that the diversity of $\beta\gamma$ structure might encode additional receptor selectivity in the contact of the trimeric $\alpha\beta\gamma$ structure. Our test with bovine rhodopsin and two distinct $\beta\gamma$ dimers, the retinal G-protein $\beta_1\gamma_1$ dimer and a distinct human placental dimer composed of $\beta_2\gamma_6$ proteins [14,15], suggested that the $\beta\gamma$ dimer made a separate and selective binding contact with rhodopsin [16]. Preparations of G$\beta\gamma$ derived from bovine brain have been resolved into fractions of defined β and γ compositions and these also show distinct affinities for rhodopsin interaction [17]. Further, we have found a marked difference between the affinities of $\beta_1\gamma_1$ and $\beta_1\gamma_2$ for bovine rhodopsin [18]. Recently, differences have been described

Abbreviations used: ConA, concanavalin A; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide; GTP[S], guanosine 5′-[γ-thio]triphosphate; ROS, rod outer segment; SPR, surface plasmon resonance.

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for the G$\beta\gamma$ selectivity of A1 adenosine receptors [19,20], the bombesin receptor family [21] and α_2 adrenergic receptors [22]. All of these investigations used the receptor catalysis of GTP exchange on the Gα subunit or the stabilization of high-affinity agonist binding to characterize the interaction of G$\beta\gamma$; they did not directly assess the binding interaction of the G$\beta\gamma$ with receptor.

The binding interactions of transducin subunits with rhodopsin have been examined with preparations of rod outer segment (ROS) discs by co-sedimentation [23] and with purified rhodopsin by using tryptophan fluorescence [24]. Both of these investigations revealed a binding of retinal $\beta_1\gamma_1$ with rhodopsin. However, neither of these studies examined $\beta\gamma$ dimer selectivity of rhodopsin or the relative binding affinities of α and $\beta\gamma$. To explore this question further, we designed procedures for the immobilization of rhodopsin to analyse G-protein subunit interactions with the technique of surface plasmon resonance (SPR) [25,26]. SPR spectroscopy offers the opportunity to make quantitative measurements of the specificity, kinetics and affinities of protein–protein interactions in real time without some of the complexities of other methods. SPR spectroscopy is a biosensor-based method that observes the interaction of one or more proteins in a mobile phase with one or more specific sites immobilized at the sensor surface. The detection principle is based on changes in the optical properties of a surface layer with increasing mass of adsorbed protein. In brief, the biosensor measures the angular dependence of SPR excitation produced by light in total internal reflection. This allows the measurement of refractive index changes of the solution within the evanescent field of the reflected light in proximity to the sensor surface. By compensating for refractive index changes of the mobile phase, the SPR signal directly reflects changes in the amount of surface-bound protein. The signal output from the SPR detector is termed a 'sensogram'; 1 'resonance unit' of the signal corresponds to approx. 1 pg of protein/mm^2 of surface. Although SPR spectroscopy was initially introduced to examine the interactions of antibodies and antigens in solution [25], the technique is becoming increasingly popular for investigating the interaction of various components of G-protein-mediated signal transduction systems [27] (for a general introduction to SPR biosensors see [28]). Here we provide evidence for the independent binding interactions of α and $\beta\gamma$ subunits with immobilized rhodopsin. The binding properties that we have measured directly with SPR replicate those inferred from biochemical studies *in vitro*. Further, SPR reveals marked differences in the kinetics of $\beta_1\gamma$ dimer association and dissociation with rhodopsin, depending on the γ-subunit chain.

EXPERIMENTAL

Preparation of G-protein subunits

Heterotrimeric transducin was prepared by the method of Kühn [29] by GTP extraction of ROS discs that were isolated by the method of Papermaster and Dreyer [30]. The α_t and $\beta\gamma$ subunits of transducin were separated by sequential chromatography over ω-amino-octylagarose (Sigma) and Blue Sepharose CL-4B (Pharmacia) as described previously [16,31]. The recombinant $\beta_1\gamma_2$ and $\beta_1\gamma_{8\text{-olf}}$ dimers were expressed in Sf9 cells by the co-infection of viruses encoding Gβ_1 and γ_2 or $\gamma_{8\text{-olf}}$ and purified as described previously [18], with additional chromatography over FPLC SuperDex HR-75 (Pharmacia). The resolved α_q subunit from the cephalopod retinal G-protein was prepared from *Sepia officinales* retina obtained from the National Resource Center for Cepholopod Molluscs, University of Texas Marine Biomedical Institute (Galveston, TX, U.S.A.) by the methods described for squid retinal G-protein [32]. For SPR analysis, $\beta_1\gamma_2$ and $\beta_1\gamma_{8\text{-olf}}$ were each exchanged into the SPR running solution [running solution A: 50 mM Mops (pH 7.5)/150 mM NaCl/3 mM $MgSO_4$/10 μM $CaCl_2$/10 μM $MnCl_2$] supplemented with 8 mM CHAPS by chromatography over Sephadex G-50 (Pharmacia); bovine retinal α_t and $\beta_1\gamma_1$ and *Sepia* Gα_q were exchanged into solution A with no detergent. Guanosine 5′-[γ-thio]triphosphate (GTP[S])-bound Gα_t was prepared by the incubation of 10 μM resolved α_t-GDP and 6 μM rhodopsin in urea-washed ROS discs with 30 μM GTP[S] for 2 h at 30 °C. ROS discs were then sedimented at 14000 ***g*** in a Heraeus Biofuge Fresco microcentrifuge for 30 min; the supernatant was filtered over Sephadex G-25 (Pharmacia) in solution A to remove unbound nucleotide and to exchange solutions for SPR.

Urea-washed ROS discs were prepared under ambient illumination as described previously [16], resulting in a mixture of meta-II and meta-III rhodopsin. Preparations of $\beta_1\gamma_{8\text{-olf}}$ were generously provided by Dr Nick Ryba (National Institute of Dental and Craniofacial Research, Bethesda, MD, U.S.A.). Protein concentrations were determined by the Amido Black method of Schaffner and Weissmann [33].

Coupling of concanavalin A (ConA) and rhodopsin to a CM-dextran sensor chip

Most SPR experiments were conducted on a BIACore apparatus (Pharmacia Biosensor) upgraded to BIACore 1000. Several experiments were also conducted with a BIACore 2000 instrument generously made available by Dr Claude Klee (National Cancer Institute, Bethesda, MD, U.S.A.). To couple rhodopsin to the CM-dextran matrix, ConA was first immobilized by amine linkage to the dextran. Carboxy groups were activated by the injection, for 7 min, of a mixture of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDC) and 0.05 M sulpho-*N*-hydroxysuccinimide, followed by the injection, for 10 min, of 0.2–0.6 mg/ml ConA (diluted in 100 mM sodium acetate, pH 4.8) for 20 min before the injection. At the termination of the ConA coupling, 1 M ethanolamine was injected for 5 min to react with any remaining activated carboxy groups. Rhodopsin was then bound to the ConA-modified surface as follows: 100 μM rhodopsin in urea-washed ROS discs was extracted with 25 mM CHAPS in 10 mM Mops, pH 7.5, at 4 °C for 30 min. The extracted ROS discs were diluted 1:4 with 50 mM Mes, pH 6.0, containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$, then injected over the ConA-modified surface at a flow rate of 2 μl/min for 25 min. After the injection, the system was washed for 30–60 min at a flow rate of 5 μl/min with running solution A to establish a stable baseline. This was essential because the ConA tetramer dissociation to dimer was often incomplete before the immobilization of rhodopsin. All subsequent injections were performed at a flow rate of 5 μl/min in the running solution. The hydrophobic G$\beta\gamma$ dimers were each prepared in a solution containing 8 mM CHAPS; these samples were diluted with running solution A to a CHAPS concentration of less than 400 μM for injection.

Solution coupling of ConA and rhodopsin to free CM-dextran resin

CM-Sephadex C-25 (Pharmacia) resin (0.5 g) was hydrated and then washed repeatedly with 100 mM Mes (pH 5.0). The resin was sedimented by gentle centrifugation and the supernatant was removed. Typically, the resin expanded to approx. 5 ml on hydration. The resin (1–2 ml) was then incubated with an equal volume of EDC (30–60 mg/ml in 100 mM Mes, pH 5.0) followed by the same volume of ConA (2 mg/ml in 100 mM Mes, pH 5.0). After 1 h of incubation at room temperature, the resin was

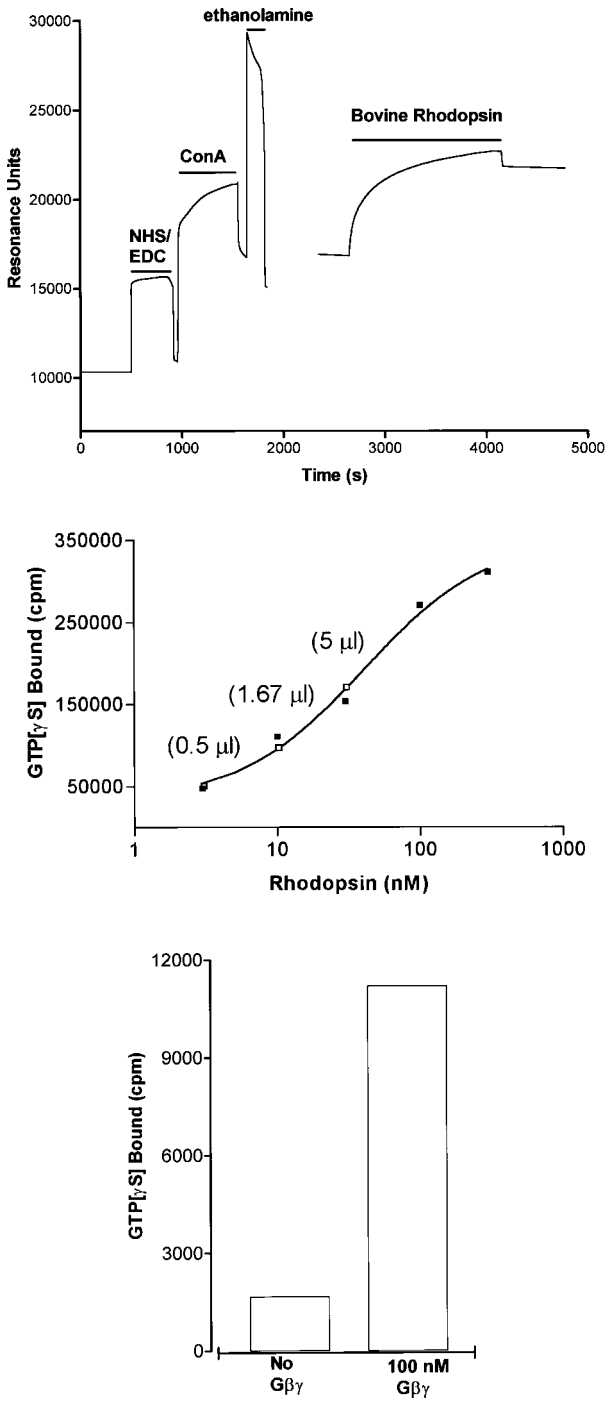

Figure 1 Immobilization of rhodopsin by binding to ConA

Top panel: sensogram recorded during the coupling of ConA by amine linkage to the CM-dextran of the sensor chip. The immobilization reactions were conducted as described in the Experimental section. The bars indicate the sequential injections of the *N*-hydroxysuccinimide/carbodi-imide (NHS/EDC) mixture to activate the carboxyls of the dextran, followed by ConA and ethanolamine to passivate unreacted activated carboxyl groups. The binding of detergent-solubilized rhodopsin to the immobilized ConA is shown with the last injection. Middle panel: analysis of the functional activity of the immobilized rhodopsin to catalyse GTP[S] exchange on α_t by using trimeric transducin. The GTP[S] exchange activities for 0.5, 1.67 and 5 μl of resin per 50 μl assay (indicated in parentheses) of the bead-immobilized rhodopsin have been fitted to a set of data for the activity of the ROS disc membranes determined in the same experiment. The specific activity of the rhodopsin coupled to the resin was calculated after PAGE analysis of the rhodopsin content of the resin assessed against ROS disc membrane standards. GTP[S] exchange assays were performed for 10 min at 30 °C in the presence of 100 nM $G\alpha_t\beta\gamma$ and 1 μM GDP as described in the Experimental section. The results are averages for duplicate determinations in a representative experiment that was reproduced five times. Bottom panel: analysis of $\beta\gamma$ dependence of the immobilized rhodopsin for catalysis of GTP[S] exchange on α_t. The GTP[S] exchange assay was performed for 10 min at 30 °C for 1 μl of resin in the presence of 500 nM α_t and 1 μM GTP[S] with or without 100 nM bovine brain $\beta\gamma$ as described in the Experimental section. Results presented are averages of duplicate determinations obtained in a representative experiment that was reproduced three times.

sedimented and resuspended for 10 min in 6 ml of ethanolamine (0.1 M). After sedimentation, the resin was then resuspended three times, 5–10 min each instance, in 5 ml of solution B [10 mM Mops (pH 7.5)/100 mM NaCl/3 mM $MnCl_2$/3 mM $CaCl_2$] at room temperature, followed by two more washes in 5 ml of solution A at 4 °C. ROS discs (approx. 100 μM, 250–300 μl) were extracted on ice for 10 min with 0.1 M CHAPS (125–150 μl). Solution A (4 ml) was added to the extracted discs and incubated for an additional 20 min on ice. The extracted ROS solution was then added to the washed resin and the mixture was rocked gently at 4 °C for 12–18 h. The final resin was then sedimented and washed five times with solution B as described above.

SDS/PAGE of proteins released from resin

After the coupling of ConA with and without rhodopsin to CM-dextran resin and extensive washing with buffer A, 30 μl of sedimented resin was mixed with 30 μl of 2 × Laemmli buffer and incubated for 20 min at room temperature to release non-covalently bound proteins from the resin. A portion of the supernatant (25 μl) was then run on a 12 % (w/v) polyacrylamide gel (Novex).

GTP[^{35}S] binding assay

Nucleotide binding assays were performed with modifications of the methods of Fawzi et al. [16] and Northup et al. [34]. GTP[S] binding to α_t was performed in a final volume of 50 μl in a solution containing 20 mM Mops, pH 7.5, 3 mg/ml BSA, 3 mM $MgSO_4$, 1 mM EDTA, 1 mM dithiothreitol, 1 μM GTP[S] or GDP, and trace amounts of GTP[^{35}S] (0.1–0.4 μCi; New England Nuclear). Reaction tubes were incubated at 30 °C for 2–10 min, stopped by the addition of 2 ml of ice-cold wash solution [20 mM Tris/HCl (pH 8.0)/25 mM $MgCl_2$/100 mM NaCl], filtered with six washes over HAWP nitrocellulose membrane filters (Millipore). Radioactivity retained on the dried filters was quantified by liquid-scintillation counting.

RESULTS

To examine the protein–protein interactions of rhodopsin and G-protein subunits, we devised procedures for the immobilization of functionally active rhodopsin for SPR studies. Figure 1 (top panel) shows the strategy for immobilization that was used in these studies. Our approach was to provide a coupling interaction of the rhodopsin molecules that would uniformly immobilize the receptor in an orientation that allowed the cytoplasmic surfaces of the receptor access to G-protein, without chemically modifying the receptor in a way that would impair G-protein interaction. We therefore used the interaction of mitogenic lectins with the extracellular carbohydrates that are known to modify receptors of the G-protein-coupled receptor class. In this experiment and in all experiments in this report, we have used ConA to bind the α-Man/α-Glc-containing carbohydrates on the extracellular portion of rhodopsin. The sensogram presented in Figure 1 (top

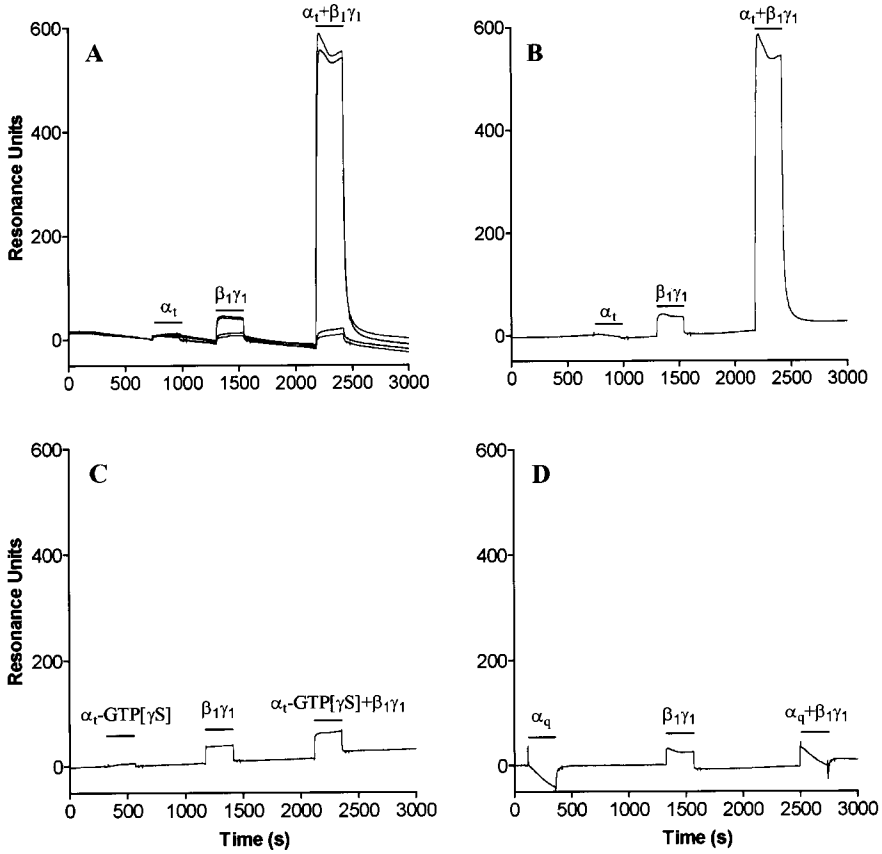

Figure 2 Synergistic interactions of the retinal G-protein subunits with immobilized rhodopsin

SPR analysis of the binding of isolated α_t, α_q and $\beta_1\gamma_1$ and their combination was examined by sequential injections of the resolved subunits and their combinations. (**A**) Four sensograms obtained from the injections of 480 nM α_t-GDP, 650 nM $\beta_1\gamma_1$ and their combination over two surfaces modified with ConA and two surfaces additionally bound with bovine rhodopsin as described in the Experimental section. (**B**–**D**) Rhodopsin-specific SPR signals from injections obtained by subtraction of the averaged SPR signals for the ConA-modified flow paths from the averaged SPR signals for the rhodopsin-bound flow paths: (**B**) the data from (**A**) presented as the rhodopsin-specific SPR signal; (**C**) the rhodopsin-specific SPR signals obtained from the injection of 480 nM α_t-GTP[S], 650 nM $\beta_1\gamma_1$ and their combination over these same four flow paths; (**D**) the rhodopsin-specific SPR signals obtained for the injection of 480 nM α_q-GDP, 650 nM $\beta_1\gamma_1$ and their combination over the four flow paths.

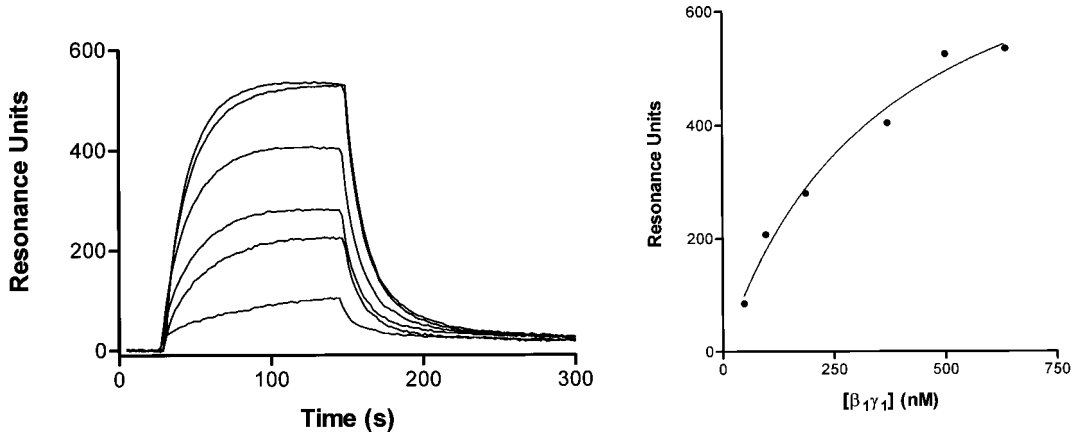

Figure 3 Saturation of synergistic binding of $\beta_1\gamma_1$-α_t to rhodopsin

Left panel: sensograms obtained for the synergistic binding of 1 μM α_t tested in the presence of six concentrations of $\beta_1\gamma_1$ from 48 to 640 nM (48, 97, 187, 370, 500 and 635 nM). For each condition the α_t and $\beta_1\gamma_1$ were mixed at 25 °C immediately before injection. The injections were performed for 120 s to ensure equilibrium values for the SPR signals. Right panel: plot of the amplitude of the plateau SPR signal against the concentration of $\beta_1\gamma_1$. The curve drawn is the best-fit curve to a single-site binding equation with the program GraphPad Prism.

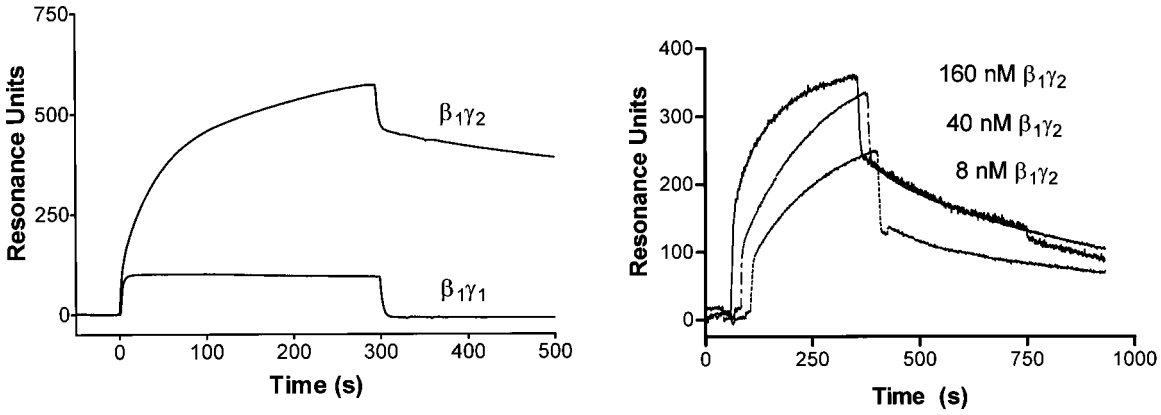

Figure 4 Selective binding of G$\beta\gamma$ subunits to rhodopsin

Left panel: comparison of the binding interactions of $\beta_1\gamma_1$ and $\beta_1\gamma_2$ dimers. Each dimer was diluted to 240 nM with the running solution with a final concentration of 400 μM CHAPS at 25 °C immediately before injection. The refractive index difference between the CHAPS-containing solution and the running solution is the cause of the rapid changes in SPR signals at the initial phases of the binding and dissociation reactions. The sensograms for the injections have been superimposed to compare the SPR signals. Right panel: kinetics of the α_t-independent binding of $\beta_1\gamma_2$ to rhodopsin. Three injections were performed with the $\beta_1\gamma_2$ diluted to concentrations ranging from 8 to 160 nM in a final CHAPS concentration of 400 μM. The sensograms for the three injections are superimposed for comparison of the SPR signals.

panel) shows the immobilization of the ConA dimer to the CM-dextran polymer in the hydrated gel of the BIACore apparatus and the subsequent binding of a solubilized rhodopsin fraction made by the detergent dispersal of urea-washed ROS disc membranes. Rhodopsin that had been coupled in a similar manner to CM-Sephadex C-25 in independent experiments was found to recover most of the catalytic activity measured in biochemical assays *in vitro* of GTP[S] exchange on the retinal G-protein. This result is presented in Figure 1 (middle panel), which compares the activity of rhodopsin in the ROS discs with that of rhodopsin that had been immobilized on CM-dextran by absorption to immobilized ConA. Independent scanning densitometry of the Coomassie Blue stain for SDS/PAGE analysis of the immobilized rhodopsin allowed us to estimate the catalytic activity of this immobilized rhodopsin at approx. $80 \pm 36\%$ (mean $\pm$ S.D., $n = 5$) of the initial activity for rhodopsin in the native ROS disc structure. When such immobilized rhodopsin was analysed for subunit-selective interaction for the turnover of nucleotide binding on the α_t subunit, the receptor displayed characteristic dependence on the $\beta\gamma$ subunit (Figure 1, bottom panel). These results strongly suggest that rhodopsin immobilized in this manner preserved both the catalytic specific activity found for rhodopsin in the native ROS disc structure and the synergistic dependence of α_t and $\beta\gamma$ subunits for the catalytic interaction.

We therefore used these procedures to analyse the binding of retinal G-protein subunits to rhodopsin. The experiments shown in Figure 2 were designed to validate the binding interactions measured by SPR for immobilized rhodopsin. A BIACore 2000 instrument, which allows simultaneous data acquisition from all four flow paths, was used to determine the contributions to the SPR signal from potential interactions of G-proteins with ConA in comparison with rhodopsin. All four flow paths were modified with ConA and two of these were additionally used to capture rhodopsin. Each sample injection was flowed through all four paths; a resulting sensogram is shown in Figure 2(A), which presents the sequential independent injections of 0.48 μM α_t and 0.65 μM $\beta_1\gamma_1$ and the injection of a mixture of both subunits at the same concentrations. These results reveal a profound synergistic binding interaction of the subunits with rhodopsin with little or no binding interactions of the G-protein subunits to immobilized ConA. The SPR signals from the two ConA-modified reference flow paths represent both non-specific binding of the G-proteins to the ConA surface and the resonance signal due to the increased refractive index of the solutions containing differing protein concentrations. These contributions to the SPR signal from the rhodopsin surfaces can be removed by subtracting the reference flow path signals from the rhodopsin signals. Figures 2(B)–2(D) present the rhodopsin-specific SPR signal obtained as the average of the SPR signals from the two rhodopsin surfaces from which the average of the SPR signals from the ConA-modified reference surfaces has been subtracted. Figure 2(B) presents the rhodopsin-specific signal from the data of Figure 2(A). Whereas 0.65 μM $\beta_1\gamma_1$ produced a rhodopsin-specific binding signal of 41 resonance units and 0.48 μM α_t a signal of 2 resonance units, their mixture resulted in a more than additive signal of 583 resonance units. We believe that the enhanced binding interaction is the basis for the synergistic enhancement of GTP[S] binding that we and others have reported previously [3,4,16]. Figure 2(C) shows that the SPR-measured binding synergy was eliminated if the α_t subunit was bound with GTP[S]. Further, the cephalopod retinal G-protein, a Gα_q-GDP species that is not activated by bovine rhodopsin in concert with bovine retinal $\beta_1\gamma_1$, did not show rhodopsin-specific binding in this experiment (Figure 2D). These results demonstrate that the SPR signals obtained for bovine rhodopsin immobilized via ConA attachment display appropriate specificity for Gα species and conformation. Conformational specificity of these interactions was further tested by denaturing the proteins. When the G-protein subunits had been denatured by incubation at 60 °C for 10 min or by treatment with 10 mM *N*-ethylmaleimide, or when the rhodopsin-coupled surface was exposed to 2% (w/v) SDS for 10 min, no synergistic binding interactions were detected (results not shown).

Two other features of the binding of the bovine retinal G-protein subunits are also shown in Figure 2. First, Figures 2(A) and 2(B) establish that at the 0.65 and 0.48 μM concentrations tested, there is an independent binding interaction of the retinal $\beta_1\gamma_1$, but not α_t, to rhodopsin. It is also readily apparent that the rates of both the association and dissociation reactions were not well determined at the time resolution of this experiment,

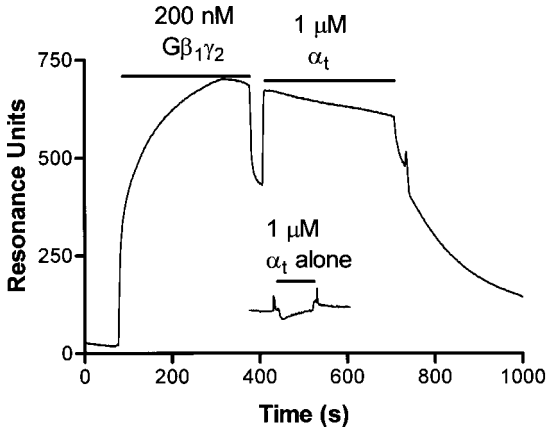

Figure 5 Sequential binding of G$\beta\gamma$ and α_t to rhodopsin

The sequential binding of $\beta_1\gamma_2$ and α_t was examined by three consecutive injections of subunits. Initially, a 2 min injection of 1 μM α_t was performed to establish the $\beta\gamma$-independent binding of α_t. Then 200 nM $\beta_1\gamma_2$ was injected in the presence of 400 μM CHAPS. After the $\beta_1\gamma_2$ injection had terminated, 1 μM α_t was again injected for 5 min. The initial 1 μM α_t injection sensogram has been superimposed on the second injection of 1 μM α_t to compare the SPR signal amplitudes.

particularly those of the independent subunits, so that kinetically determined equilibrium binding constants were not obtained for these proteins. However, by using the plateau values of binding it is possible to estimate an equilibrium dissociation constant for $\beta_1\gamma_1$ in the presence of α_t. On the basis of the synergism shown above, we designed experiments to measure the affinity of $\beta_1\gamma_1$ for binding to rhodopsin. Figure 3 (left panel) shows an overlay of the SPR traces from the independent injections of various concentrations of the $\beta_1\gamma_1$ dimer together with a constant 1 μM concentration of the α_t subunit. Figure 3 (right panel) plots the plateau values of the SPR signal as a function of the $\beta_1\gamma_1$ concentrations injected. These data were well fitted by a single-site binding model, yielding an equilibrium dissociation constant of approx. 350 nM. Independent replications of this experiment yielded a K_d of 336 ± 171 nM (mean $\pm$ S.D., $n = 3$) under these conditions. These results are in good agreement with the value of 254 ± 21 nM obtained for the facilitation by $\beta_1\gamma_1$ of rhodopsin-catalysed GTP[S] binding to α_t [16].

The independent binding interaction of $\beta_1\gamma_1$ with rhodopsin in Figures 2(A) and 2(B) confirmed our hypothesis of such a contact as the basis for the increased apparent affinity of rhodopsin for α_t in the presence of $\beta\gamma$ subunits [16]. We have also hypothesized that differences in the direct binding interactions of $\beta\gamma$ subunits with distinct protein compositions were the bases for differences in the apparent affinities based on rhodopsin-catalysed GTP[S] binding to α_t. The experiments in Figure 4 examined the selectivity of the independent binding interactions of two distinct $\beta\gamma$ dimer preparations with rhodopsin. In this experiment, $\beta_1\gamma_1$ and $\beta_1\gamma_2$ were each tested for rhodopsin binding at 240 nM in the absence of α. What is immediately evident from these data is that the two dimers displayed widely different kinetics both of association and dissociation from rhodopsin. Whereas the retinal $\beta_1\gamma_1$ equilibrated quite rapidly, as seen previously, the $\beta_1\gamma_2$ dimers equilibrated more slowly and dissociated much more slowly than $\beta_1\gamma_1$. In other experiments, preparations of $\beta_1\gamma_{8\text{-olf}}$ displayed kinetics of association and dissociation that were similar to those of the $\beta_1\gamma_2$ dimer (results not shown). From experiments such as those shown in Figure 4 (right panel), we can use the association and dissociation rate constants to calculate an equilibrium constant of 13 ± 3 nM (mean $\pm$ S.D., $n = 3$) for the $\beta_1\gamma_2$ protein. This is consistent with the value 10 nM that we obtained for $\beta_1\gamma_2$ in the GTP[S] binding assay [18]. This experiment showed that the dissociation phase, as expected, was a concentration-independent parameter, whereas the association rate varied with the free concentration of $\beta_1\gamma$.

The difference in the dissociation rate constant of $\beta_1\gamma_1$, which was not well resolved in our experiments (half-time less than 5 s), and those of the other $\beta\gamma$ dimers tested, which had dissociation half-times on the order of 600–1000 s at 25 °C, was marked. We took advantage of this slow dissociation of $\beta_1\gamma_2$ to examine the potential for sequential binding interactions of α_t and $\beta\gamma$ in the experiment shown in Figure 5. In this experiment an initial

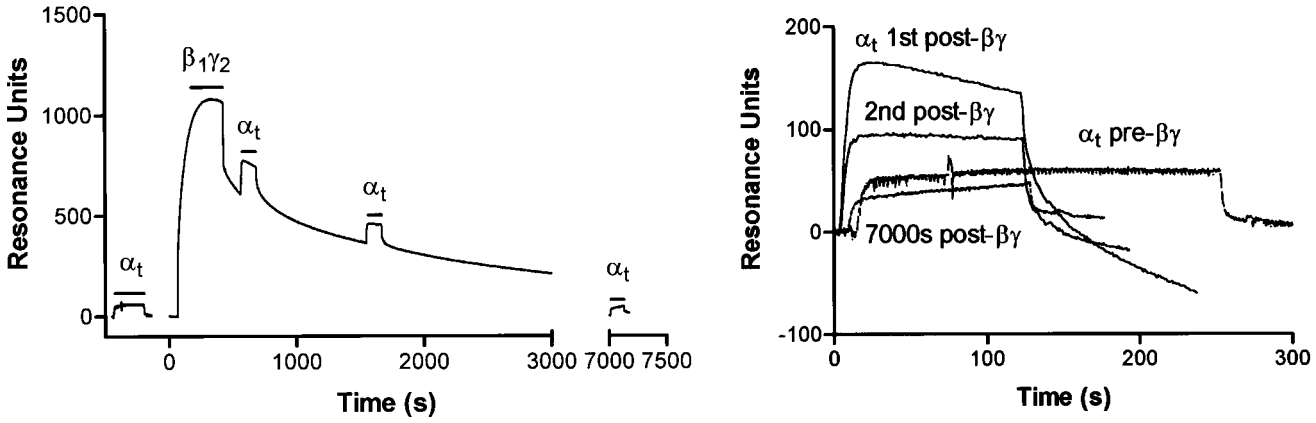

Figure 6 G$\beta\gamma$ dependence of the binding of α_t to rhodopsin

Left panel: the influence of $\beta_1\gamma_2$ saturation of rhodopsin on α_t binding was examined in four sequential injections of α_t. α_t (3 μM) was injected before the injection of 240 nM $\beta_1\gamma_2$ with 400 μM CHAPS and at the indicated times during the dissociation of the $\beta_1\gamma_2$ from rhodopsin. A second injection of 3 μM α_t was made immediately after the injection of $\beta_1\gamma_2$, after the flow path had re-equilibrated to the running solution to remove the refractive index change contributed by the CHAPS. A third injection of 3 μM α_t was performed at approximately half-dissociation of the $\beta_1\gamma_2$. The final injection of 3 μM α_t was made approx. 7000 s after the injection of $\beta_1\gamma_2$, at which time the dissociation of $\beta_1\gamma_2$ from rhodopsin was essentially complete. The initial α_t injection lasted for 240 s; all subsequent injections were for 120 s. Right panel: an overlay of the SPR signals for each of the four α_t injections. The sensograms for each of the α_t injections have been baseline-adjusted to the value immediately before injection; they are superimposed for comparison of the SPR signals.

injection of 1 μM α_t in the absence of $\beta\gamma$ confirmed the absence of a detectable binding interaction. Rhodopsin was then saturated by injection of 200 nM $\beta_1\gamma_2$ for 5 min. The subsequent injection of 1 μM α_t then produced a greatly increased SPR signal. Because all of the unbound $\beta_1\gamma_2$ would have been removed from the flow path in the interval between the two injections, we conclude that α_t and $\beta\gamma$ were able to bind independently and sequentially to rhodopsin. The synergy identified in the experiment shown in Figure 5 was comparable to that found for the co-injection experiments of Figures 2(A) and 3 (left panel). To determine the quantitative relationship between the fractional saturation of rhodopsin by $\beta_1\gamma_2$ and the synergistic binding of α_t, we performed the experiment presented in Figure 6 (left panel). In this experiment we examined first the injection of a 3 μM concentration of the retinal α subunit, independently of $\beta\gamma$, followed by the injection of 240 nM $\beta_1\gamma_2$ to reach saturation, then three further independent injections of 3 μM α_t subunit chain during the course of the dissociation of $\beta_1\gamma_2$ from rhodopsin. As shown in Figure 6 (right panel), the initial injection of α_t after the formation of the rhodopsin–$\beta_1\gamma_2$ complex attained the highest SPR signal for the α_t injection. A subsequent injection, initiated at approximately half-dissociation of the bound $\beta_1\gamma_2$, revealed that the SPR signal diminished by approximately half compared with the previous α_t injection. A final injection made approx. 7000 s after the injection of the $\beta_1\gamma_2$, at which time all $\beta_1\gamma_2$ signal had dissociated to baseline, shows an SPR signal for the α_t that was nearly identical with that of the injection before $\beta_1\gamma_2$. It should be noted that in each instance of α_t subunit injection, the approach to equilibrium binding occurred essentially within the mixing time of the flow cell, so that kinetics of the association rate was not obtained. The α_t subunit also dissociated rapidly, although in the initial α_t injection after $\beta_1\gamma_2$ the dissociation of the $\beta_1\gamma_2$ was combined with that of α_t. Subsequent injections of the α_t showed rapid dissociation as seen for α_t alone, so that in each case the interaction of α_t subunit with rhodopsin was promptly equilibrated, i.e. rapidly associating and rapidly dissociating. What is quite apparent from this experiment is that the binding of α_t is quantitatively linked to the binding of $\beta_1\gamma_2$.

DISCUSSION

We conclude from these studies that the strategy of immobilizing rhodopsin through the interaction of a mitogenic lectin with extracellular carbohydrates succeeded in producing a preparation of biochemically active rhodopsin that was appropriately oriented for interaction with G-protein subunits. Such immobilized rhodopsin provides a useful reagent for the measurement of G-protein subunit interactions with the receptor. Because the extracellular carbohydrate modification is a common feature of G-protein-coupled receptors, it is reasonable to expect that a similar coupling strategy could be successful for other G-protein-coupled receptors that can be expressed and isolated to homogeneity. We have found, as with rhodopsin, that other G-protein-coupled receptors in the native membrane environment are resistant to denaturation by chaotropic agents and are suitable for many of the biochemical techniques developed to study visual transduction to identify selective biochemical interactions of G-protein subunits with those receptors [21,32,35]. Such studies, in combination with SPR studies, would be an approach for a further examination of the hypothesis that $\beta\gamma$ dimers encode selective receptor contacts.

In our present work, the SPR measurements reveal that the kinetics of the interactions of the retinal α_t and $\beta_1\gamma_1$ subunits with rhodopsin is extremely rapid, essentially outside the resolution time of this instrument to measure. This rapidity was expected for the catalytic interaction of rhodopsin with the retinal G-protein. Because the product of the catalysis was the dissociated α_t subunit, these results are consistent with biochemical data from ROSs and isolated ROS discs *in vitro*, demonstrating the rapidity of the rhodopsin signalling [36,37]. Although the BIACore was unable to resolve the kinetics of retinal α_t and $\beta_1\gamma_1$ binding interactions with rhodopsin, and neither retinal subunit alone bound to rhodopsin with high affinity, the addition of the two proteins together clearly identified a synergistic binding interaction of the two subunits with rhodopsin. This is in agreement with our initial examination of the thermodynamics of rhodopsin–G-protein interaction that revealed an approx. 60-fold enhancement of α_t subunit binding interaction by the $\beta\gamma$ subunit [16]. Further, the K_d for $\beta_1\gamma_1$-rhodopsin binding in the presence of α_t measured by SPR was in good agreement with the $K_{1/2}$ obtained for the $\beta_1\gamma_1$ facilitation of rhodopsin-catalysed GTP[S] binding to α_t [16]. These data argue that the receptor-catalysed exchange assay might provide a good estimate for the binding interactions of receptors with $\beta\gamma$ dimers.

This conclusion was examined more directly by using the binding of the non-retinal $\beta_1\gamma$ dimers. Here the interactions of the heterologous $\beta_1\gamma$ subunit dimers with rhodopsin showed markedly different kinetics from the retinal $\beta_1\gamma_1$ dimer. In addition, both $\beta_1\gamma_2$ and $\beta_1\gamma_{8\text{-olf}}$ dimers displayed a significant binding affinity for rhodopsin independently of α_t. Further, the kinetically determined K_d for the α_t-independent binding of $\beta_1\gamma_2$ agreed well with the $K_{1/2}$ obtained from the rhodopsin-catalysed exchange assay, providing additional confirmation of the validity of the latter to measure receptor-$\beta\gamma$ binding affinities. As found for the biochemical assay [16–18,38] the α_t-independent binding of $\beta\gamma$ to rhodopsin is strongly influenced by the γ subunit. While retinal $\beta_1\gamma_1$ associated and dissociated too rapidly for kinetic evaluation in our studies ($t_{1/2} < 5$ s), the $\beta_1\gamma_2$ and $\beta_1\gamma_{8\text{-olf}}$ dimers each associated more slowly and displayed markedly prolonged binding lifetimes ($t_{1/2} > 600$ s). The dissociation of $\beta_1\gamma_2$ was in agreement with other data obtained in our laboratory with biochemical analysis *in vitro* for the facilitation of GTP/GDP exchange reaction with the α_t subunit (W. A. Clark, unpublished work). It is unlikely that the binding interactions that we measured with SPR are determined entirely by non-specific hydrophobic interactions directed by the isoprenoid moiety of the Gγ chain, because denaturing rhodopsin abolished the binding of the subunits. Further, the binding of farnesylated G$\beta_1\gamma_1$ is strongly dependent on the additional protein–protein interactions of Gα_t and is conformationally sensitive to the guanine nucleotide bound to the Gα_t subunit. It remains to be determined whether the differences we have measured in the Gα_t-independent binding between $\beta_1\gamma_1$ and $\beta_1\gamma_2$ are due solely to the isoprenoid modifications, as has been suggested [39], or arise from distinct protein recognition of the Gγ chain by rhodopsin.

The slow dissociation of $\beta_1\gamma_2$ from rhodopsin permits the examination of the α_t subunit interaction, both independent of and dependent on $\beta\gamma$. In all conditions, the α_t subunit interaction equilibrated within the mixing time of the flow cell and the dissociation was equally rapid. However, the amplitude of the α_t binding signal varied in direct proportion to the $\beta_1\gamma_2$–rhodopsin binding: immediately after $\beta_1\gamma_2$–rhodopsin binding equilibrium was attained, the α_t binding signal was maximal; at approximately half dissociation of $\beta_1\gamma_2$ the α_t binding signal was about half-maximal; and on complete dissociation of $\beta_1\gamma_2$ the α_t binding was minimal. These results parallel the binding synergism found for retinal $\beta_1\gamma_1$ with α_t. They suggest further that the binding interactions might be sequential. The recent kinetic evaluation of the rhodopsin–transducin interactions that suggest

a two-step mechanism [40] might be interpreted in the light of our results as suggesting a two-step binding interaction, with $\beta_1\gamma_1$ contact preceding the interaction of α_t with rhodopsin.

Our previous data from rhodopsin–transducin activation studies [16] led us to predict, on the basis of thermodynamic arguments, an independent and selective binding contact of $\beta_1\gamma_1$ with rhodopsin. The results that we have obtained with SPR signals in the BIACore instrument measuring rhodopsin–$\beta_1\gamma_1$ interactions seem to confirm that prediction. Our current results also extend the observations made by other laboratories after the initial observations of the requirement for the $\beta_1\gamma_1$ dimer in the activation of α_t by rhodopsin [3,4]. These latter experiments include the sedimentation of $\beta_1\gamma_1$ together with urea-washed ROS discs [23] and $\beta_1\gamma_1$-elicited changes in tryptophan fluorescence in liposome-incorporated rhodopsin [24]. Two other laboratories have used SPR approaches to measure rhodopsin–transducin interactions in bilayers [41] or hexagonal array-packed lipid structures [42]. These studies demonstrated light-regulated changes in the apparent equilibrium dissociation constants for retinal G-protein with rhodopsin. Our current study circumvents the limitations of these previous studies imposed by the amphipathic properties of the retinal G-protein subunits, which displayed significant binding interactions with the lipid matrices employed for rhodopsin presentation. Furthermore, we have been able to examine directly the kinetics and $\beta\gamma$-selectivity of the binding interactions with rhodopsin.

What emerges from our kinetic examination is that the retinal α_t and $\beta_1\gamma_1$ subunit interactions are transient in comparison with the heterologous $\beta\gamma$ dimers. This raises the possibility that in addition to conferring selectivity for specific receptors, the diversity of $\beta\gamma$ gene products might also enable receptor–$\beta\gamma$ interactions with distinct kinetic properties. Finally, although the biological significance is certainly not established, our results demonstrate that the interactions of rhodopsin with G-protein subunits can be sequential. If this were true in biological systems, it would suggest that the interaction of G-proteins with receptor need not proceed via the heterotrimer. Instead, complexes of activated receptor bound with G$\beta\gamma$ might cycle through non-activated Gα–GDP subunits.

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