Phosphatidylethanolamine Enhances Rhodopsin Photoactivation and Transducin Binding in a Solid Supported Lipid Bilayer as Determined Using Plasmon-Waveguide Resonance Spectroscopy

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ABSTRACT Flash photolysis studies have shown that the membrane lipid environment strongly influences the ability of rhodopsin to form the key metarhodopsin II intermediate. Here we have used plasmon-waveguide resonance (PWR) spectroscopy, an optical method sensitive to both mass and conformation, to probe the effects of lipid composition on conformational changes of rhodopsin induced by light and due to binding and activation of transducin (Gt). Octylglucoside-solubilized rhodopsin was incorporated by detergent dilution into solid-supported bilayers composed either of egg phosphatidylcholine or various mixtures of a nonlamellar-forming lipid (dioleoylphosphatidylethanolamine; DOPE) together with a lamellar-forming lipid (dioleoylphosphatidylcholine; DOPC). Light-induced proteolipid conformational changes as a function of pH correlated well with previous flash photolysis studies, indicating that the PWR spectral shifts monitored metarhodopsin II formation. The magnitude of these effects, and hence the extent of the conformational transition, was found to be proportional to the DOPE content. Our data are consistent with previous suggestions that lipids having a negative spontaneous curvature favor elongation of rhodopsin during the activation process. In addition, measurements of the Gt/rhodopsin interaction in a DOPC/DOPE (25:75) bilayer at pH 5 demonstrated that light activation increased the affinity for Gt from 64 nM to 0.7 nM, whereas Gt affinity for dark-adapted rhodopsin was unchanged. By contrast, in DOPC bilayers the affinity of Gt for light-activated rhodopsin was only 18 nM at pH 5. Moreover exchange of GDP for GTPγS was also monitored by PWR spectroscopy. Only the light-activated receptor was able to induce this exchange which was unaffected by DOPE incorporation. These findings demonstrate that nonbilayer-forming lipids can alter functionally linked conformational changes of G-protein-coupled receptors in membranes, as well as their interactions with downstream effector proteins.

INTRODUCTION

Visual phototransduction serves as an important model for many other G-protein signaling systems involving cellular membranes. Rhodopsin, the rod cell photoreceptor, is probably the best-studied G-protein-coupled receptor (GPCR) and is the only one for which high-resolution crystal structures are available (Palczewski et al., 2000; Okada et al., 2002). Activation of the receptor, by photoisomerization of the 11-cis-retinal cofactor in the case of rhodopsin, leads to conformational changes at the cytoplasmic membrane surface. The active receptor interacts with a heterotrimeric G-protein (transducin, Gt) to catalyze guanine-nucleotide exchange and initiate visual signaling. Within a few milliseconds after photon absorption, a metastable equilibrium is established between two conformational states of photoexcited rhodopsin, called metarhodopsin I (MI) and metarhodopsin II (MII), which depends on pH and temperature (Thorgerisson et al., 1993; Jäger et al., 1998). The MI form (with absorption band at λ = 478 nm) is distinguished by its protonated Schiff base linkage between the retinal and Lys296, where Glu113 functions as the counterion. In the MII conformation (with absorption band at λ = 380 nm) the retinal Schiff base is deprotonated and the salt bridge to Glu113 is broken, leading to a protein conformation change (Hubbell et al., 2003). MII is the conformation of the photoisomerized rhodopsin that binds and activates transducin. Formation of the MI state of rhodopsin is characterized by relative movements of transmembrane helices III, V, and VI, as determined by site-directed spin-labeling experiments (Hubbell et al., 2003). Binding of Gt by MII catalyzes the exchange of GDP for GTP bound to the Gt α-subunit, which dissociates and carries the visual signal from rhodopsin to the effector, cyclic GMP phosphodiesterase. The activated phosphodiesterase then catalyzes the hydrolysis of cGMP, which triggers the closure of cGMP-gated Na+ channels, yielding hyperpolarization of the rod outer segment (ROS) plasma membrane and the amplified visual response (for a review, see Menon et al., 2001).

The ratio of [MII]/[MI] after an actinic flash is indicative of the ability of rhodopsin in a lipid membrane recombinant to function properly in vitro. Interaction of rhodopsin with the surrounding lipids appears to be important for its conformation and function (Lamola et al., 1974; Baldwin and Hubbell, 1985; Wiedmann et al., 1988; Mitchell et al., 1992; Gibson and Brown, 1993; Brown, 1994; Mitchell and Litman, 1999; Botelho et al., 2002; Niu et al., 2002), perhaps by influencing the movement of helices during the activation process. When rhodopsin is reconstituted into saturated lipids, conformational changes that induce Gt binding are accompanied by significant changes in the absorption spectra of the rhodopsin moiety.

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phospholipids, the formation of MII is inhibited (Baldwin and Hubbell, 1985; Mitchell et al., 1992; Gibson and Brown, 1993; Litman and Mitchell, 1996). Flash photolysis studies have shown that the presence of nonlamellar-forming lipids such as dioleoylphosphatidylethanolamine (DOPE) favors the MII state (Wiedmann et al., 1988; Brown, 1994; Botelho et al., 2002), which is thermodynamically driven by matching the spontaneous curvature of the lipid bilayer to that of the water/lipid interface adjacent to the protein (Botelho et al., 2002). There is also lipid restructuring that occurs during the photoactivation process (Lamola et al., 1974; Hessel et al., 2001; Botelho et al., 2002), and this may also be influenced by the bilayer lipid composition.

As a means of gaining further insight into the role of the lipid environment in receptor activation, we have used plasmon-waveguide resonance (PWR) spectroscopy (Salamon et al., 1997, 1999) to monitor rhodopsin photoactivation and transducin binding and activation. This technique enables real-time characterization of mass density and conformational changes upon protein-protein, protein-lipid, and protein-ligand interactions occurring either within or at the surface of anisotropic membrane thin films. No labeling is required since the analytical method is dependent on the intrinsic optical properties (refractive index, n, and optical absorption coefficient, k), as well as the thickness, t, of thin layers of material deposited onto the resonator surface. In this way it is possible to detect as little as femtomole quantities of material. This technique has been successfully used in our laboratories as a method for the characterization of a variety of anisotropic proteolipid membranes (Salamon et al., 2000a,b; Alves et al., 2003). An important aspect is that PWR has the unique capability of independently examining changes in the optical properties (i.e., n and k) of a uniaxially oriented sample, such as a proteolipid film, both parallel and perpendicular to the membrane plane in response to receptor-ligand interactions. As a result, one can monitor changes in the refractive index anisotropy in relation to long-range molecular order and molecular conformational changes occurring in the membrane upon protein functional activity (Salamon and Tollin, 2001a). Moreover, PWR spectroscopy possesses several significant advantages compared to conventional surface plasmon resonance (SPR) methods, which have been applied in previous studies of membranes containing rhodopsin (Salamon et al., 1994, 1996). These include enhanced sensitivity and spectral resolution (due to narrower line widths) and the ability to distinguish between mass and conformational changes (Salamon et al., 1997). The latter is a consequence of the ability of PWR to use both p- and s-polarized light excitation (electric vectors perpendicular and parallel to the membrane plane, respectively) to produce resonances and thereby to permit structural characterization (Salamon and Tollin, 1999, 2001b), whereas SPR only responds to p-polarized excitation.

In the experiments included in this study, we incorporated detergent-solubilized rhodopsin into a solid-supported lipid bilayer and examined PWR spectral changes occurring upon light activation. We have investigated the influences of the lipid bilayer composition (especially the presence of non-lamellar-forming lipids such as DOPE), as well as the effect of pH on the PWR spectra. The magnitude of the spectral shifts caused by light was found to vary with pH in a manner comparable to that observed in flash photolysis experiments that directly monitor MII formation (Botelho et al., 2002), i.e., a shift in the pKa to larger values occurred when going from egg phosphatidylcholine (PC) to dioleoylphosphatidylcholine (DOPC)/DOPE (25:75 mol %) bilayers. On account of this correspondence, we are able to conclude that PWR is monitoring MII formation in our experiments. We have also observed that increasing the molar ratio of DOPE to DOPC caused an increase in the extent of the conformational changes observed in the proteolipid system. This is a new insight that could not have been obtained using flash photolysis, since the latter method follows changes in the chromophore rather than in the whole proteolipid membrane as does PWR. Our findings are consistent with the known tendency of DOPE to promote the reverse hexagonal (HII) phase (Brown, 1994), whose negative spontaneous curvature may help drive the elongation process of the receptor that exposes important sites for transducin interaction, and thereby causes the lipid movement known to occur during the light activation process (Brown, 1994; Botelho et al., 2002). Moreover, we investigated the interaction of Gt with both the dark-adapted (Rho) and light-activated (Rho*) states of rhodopsin, as well as the ability of Gt to undergo GDP-GTPyS exchange. The affinity of Gt to the receptor was found to be highly dependent on the activation state, with a higher affinity for Rho* (0.7 nM) than for Rho (64 nM) in a DOPC/DOPE (25:75 mol %) bilayer. Significant influences of the membrane environment were evident in that the affinity of Gt for Rho* was decreased to 18 nM in a DOPC bilayer. No effect of bilayer composition was observed on the affinity of Gt to Rho, or on the affinity of GDPyS to the G-protein. Thus, only the light-induced conformational transition was influenced by DOPE incorporation. These results further demonstrate that the lipid bilayer composition plays a major role in determining the activation and initial signal transduction events involved in rhodopsin visual signaling, and provide additional insights into this process. The PWR experiments establish a structural correlation with flash photolysis studies of rhodopsin (Botelho et al., 2002), and thus enable extension to other receptor systems where light activation is not feasible at present.

**EXPERIMENTAL PROCEDURES**

**Purification of retinal rod membranes and solubilization of rhodopsin**

Bovine ROS membranes containing rhodopsin were prepared from frozen retinas (Papermaster and Dryer, 1974). The procedure includes washing of the ROS membranes with hypotonic buffer; this is used to remove soluble and peripheral ROS proteins (e.g., transducin, phosphodiesterase, arrestin, ...
The purity of the preparation was assessed by sodium dodecyl sulfate gel electrophoresis (data not shown) revealing the samples to be of good purity, with a predominant rhodopsin band upon staining with Coumassie blue. The preparations typically had $A_{280}/A_{490}$ absorbance ratios of 2.5, were completely bleached by actinic illumination, and were 99% regenerable with 11-cis-retinal. ROS membranes were homogenized in 10 mM phosphate buffer, pH 6.8, containing 200 mM octylglucoside (detergent/protein ratio was $\sim$2000:1), and centrifuged at 64,000 × g for 1 h at 4°C. The detergent-solubilized rhodopsin sample was concentrated using 10 kDa cutoff centrifics (Millipore, Bedford, MA), which allowed the removal of any lipid-detergent aggregates that were present. The sample was then suspended in 10 mM phosphate buffer to a final detergent concentration of 30 mM. All rhodopsin samples were handled in dim red light (Kodak safelight filter no. 1, 15-W bulb) and kept at 4°C under an argon atmosphere when possible.

**Isolation and purification of transducin**

$G_t$ was extracted from bovine ROS membranes and purified using a method modified from that described (Fung et al., 1981). ROS membranes were maintained under light conditions from the initial step of ROS isolation and during all subsequent stages to maximize the amounts of transducin bound (Baehr et al., 1982). $G_t$ ($\sim$3 mg) was obtained from 100 retinas after hexylagarose purification. The GT-Pase activity was assayed using a phosphate assay kit (Molecular Probes, Eugene, OR) as described (Webb, 1992). The concentration of product was measured in a plate reader ($\mu$Quant, Bio-Tek Instruments, Winooski, VT) based on the absorbance at 560 nm. The GT-Pase activity of the purified $G_t$ was determined to be 5 mmol/min, in good agreement with published results (Kuhn, 1980; Fung et al., 1981). Transducin was used within 2 weeks of its preparation during which time it was stored at $-70^\circ$C in phosphate buffer in the presence of a protease inhibitor cocktail (Sigma, St. Louis, MO).

**Plasmon-waveguide resonance spectroscopy**

Resonance spectra in this study were obtained using a Beta PWR instrument from Proterion (Piscataway, NJ) that records the relative reflectance (i.e., the ratio of reflected to incident light intensities) versus the absolute incident light intensity of 360 nm. The GT-Pase activity of the purified $G_t$ was determined to be 5 mmol/min, in good agreement with published results (Kuhn, 1980; Fung et al., 1981). Transducin was used within 2 weeks of its preparation during which time it was stored at $-70^\circ$C in phosphate buffer in the presence of a protease inhibitor cocktail (Sigma, St. Louis, MO).

**Formation of solid-supported lipid bilayers**

In this study we used self-assembled solid-supported lipid membranes (Salamon et al., 1999). The method of preparation employs the same principles that govern the spontaneous formation of a freely suspended lipid bilayer membrane. This involves spreading a small amount of lipid solution across a 2-mm orifice in a Teflon block that separates the thin SiO$_2$ film on the surface of the plasmon generator from the aqueous phase. The hydrophilic surface of the hydrated silica (Gee et al., 1990; Silberzan et al., 1991) attracts the polar groups of the lipid molecules, thus forming a lipid monolayer deposited on a layer of adsorbed water, with the hydrocarbon chains oriented toward the droplet of excess lipid solution. Filling the main body of the cell sample with the appropriate aqueous solution initiates the second step, which involves a thinning process with the formation of both the second monolayer and the plateau-Gibbs border that anchors the bilayer film to the Teflon spacer, allowing the excess of lipid and solvent to move out of the orifice (Salamon and Tollin, 1999). In these experiments, the lipid films were formed on the silica surface using the following membrane-forming solution: 7 mg/mL of a single lipid or lipid mixtures in squalene/butanol/methanol (0.05:0.95:0.5, v/v). Unless otherwise noted, experiments were performed using three lipid compositions: egg PC, DOPC, or a mixture of 25 mol % DOPC and 75 mol % DOPE. The incorporation of rhodopsin into the solid-supported lipid bilayer was accomplished by introducing the octylglucoside-solubilized receptor ($\approx$1 μM final concentration of rhodopsin) into the aqueous compartment under conditions that dilute the detergent below the critical micelle concentration (25 mM). The final detergent concentration in the cell sample was $\sim$3 mM (100 μL injection of a 30-mM octylglucoside solution into a 1-mL cell sample), which is well below the detergent critical micelle concentration. Under these conditions the membrane protein spontaneously incorporated into the lipid bilayer. The aqueous compartment was filled with 1 mL of 10 mM phosphate buffer at the desired pH. Control experiments have been performed to assess the effect of octylglucoside on the lipid bilayer. This work has shown that there are no appreciable PWR resonance shifts when $<6$ mM of detergent is present in the cell sample compartment. In our experiments the maximal final detergent concentration in the cell sample was 3 mM, and so the effects on the PWR signal were negligible.
All experiments were performed using similar amounts of rhodopsin incorporated into the lipid bilayer as monitored by PWR spectral shifts obtained upon rhodopsin incorporation. We have determined that having larger amounts of rhodopsin in the lipid bilayer does not affect either the pKa values for the MI-MII transition or the binding affinity to transducin, although it does produce larger spectral shifts. It should also be noted that in these experiments we do not directly determine the concentrations of receptor and Gt in the lipid bilayer membrane. Affinities are obtained from the PWR spectral changes that occur due to mass increases in the proteolipid system upon incremental addition of ligand or Gt-protein to the aqueous cell compartment. Since PWR is only sensitive to the optical properties of material that is deposited on the resonator surface, there is no interference from the material that is in the bulk solution. Furthermore, in titrations with ligand or Gt, the amount of material bound is quite small compared to the amount present in the bulk solution, and it is assumed that the bulk material is able to freely diffuse and equilibrate with the membrane. Thus, since the spectral changes are proportional to the amount of G-protein bound to the receptor, plots of spectral shifts versus bulk G-protein concentration allow a direct determination of dissociation constants. In other words, a hyperbolic saturation curve corresponds to a plot of the total G-protein added to the aqueous compartment versus the amount bound, and fitting the experimental data to such a curve provides a thermodynamically meaningful value for Kd.

Effect of illumination on plasmon-waveguide resonance spectra

After rhodopsin was incorporated into the lipid bilayer and the PWR spectra stabilized (i.e., an equilibrium condition was reached), the aqueous compartment of the PWR cell was illuminated with yellow light (wavelength >500 nm) from a 150-W tungsten-halogen light source using a fiberoptic light guide (Postec, Auburn, NY). The cell sample was irradiated several times (total of 20 s over an interval of 5 min) and the PWR spectra were monitored until no further spectral changes were observed (i.e., until saturation was achieved). Such irradiation is known to convert rhodopsin to the MII state. The PWR spectral changes were monitored after each 1- to 2-s light exposure until the spectra were found to stabilize; this was considered to correspond to formation of the maximum amount of the MII state of rhodopsin. Inasmuch as the MI-MII equilibrium state has been reported to have a lifetime of ~15 min (Arnis and Hofmann, 1993), and the PWR spectrum was obtained within ~1–2 min after irradiation, this assumption is not unreasonable. To confirm that the MII state was being formed under these conditions, a control experiment was performed in which a sample of rhodopsin solution in the absence of a lipid bilayer was taken from the PWR cell after illumination with yellow light, and the absorption spectrum was measured. This control demonstrated that under the instrument light illumination conditions the sample was photosactivated with total conversion of the dark-adapted rhodopsin to MII. Additional previous control studies have employed the use of hydroxyamine, which traps the photoproducts as retinaloxamine plus opsin, to demonstrate photoactivation of rhodopsin in similar SPR experiments (Salamon et al., 1994). In the experiments reported below, we have taken care to add the same amount of rhodopsin to the sample cell and to carefully monitor the degree of rhodopsin incorporation based on the observed PWR spectral changes. This allowed comparisons to be made between different experiments. We should also point out that in what follows, when reference to MII formation is made, we are referring to the dynamic equilibrium between the MI/MII states known to occur upon rhodopsin light irradiation.

Interaction of transducin and GTPγS with the proteolipid system

Either before or after activating rhodopsin with light, Gt interaction with the proteolipid system was investigated by monitoring the PWR spectral changes obtained upon incremental additions of a Gt solution. Affinity constants that describe those interactions were obtained by plotting the resonance position spectral shift at different Gt concentrations, and fitting the data using a hyperbolic function that describes the interaction between two molecules (Salamon et al., 1996). Since the PWR spectral change is directly proportional to the concentration of Gt-bound receptor, and the amount of Gt bound is much smaller than the amount of unbound Gt, the Kd value can be calculated from such a hyperbolic plot without knowledge of the absolute concentrations within the proteolipid membrane. After saturation with Gt (either with dark-adapted or light-activated rhodopsin), a GTPγS solution was incrementally added to the system, PWR spectral changes were monitored, and affinities for the interaction of Gt with rhodopsin were determined as described above.

RESULTS AND DISCUSSION

PWR changes observed upon incorporation of rhodopsin into a solid-supported lipid bilayer

As shown in Fig. 1A and B, the incorporation of rhodopsin into an egg PC lipid bilayer produced shifts to higher resonance angles in the PWR spectra for both p- and s-polarized light. Such shifts in the resonance position are due to the larger refractive index and bilayer thickness that results from the increase and redistribution of mass in the proteolipid system upon receptor incorporation into the bilayer. Insertion of rhodopsin molecules into a lipid membrane also causes alteration of the lipid molecular long-range order and packing density by forcing some of the lipids out of the bilayer into the Gibbs border, thereby changing the average structure of the proteolipid membrane. This also causes changes in the PWR spectra. It should be noted that shifts in the p-polarized resonance upon receptor incorporation into the bilayer were much larger (~70 mdeg) than those obtained with s-polarization (~40 mdeg). This reflects a change in refractive index anisotropy that is characteristic of uniaxially oriented anisotropic (i.e., spherically shaped) molecules such as GPCRs, whose dimensions perpendicular to the bilayer plane are larger than in the parallel plane. Models of rhodopsin based on x-ray diffraction show an ellipsoidal structure with a length of ~75 Å in the direction perpendicular to the membrane and a width of ~48 Å (Palczewski et al., 2000). The increase in refractive index anisotropy observed here is a direct indication of the uniaxial insertion of rhodopsin into the lipid bilayer with its long axis perpendicular to the membrane plane. It should be noted, however, that the direction of insertion is not unambiguously indicated by these results.

In addition to the change in the PWR resonance positions observed in the experiments in this study, there was also an increase in the amplitude of the PWR spectra for both polarizations, especially with the s-polarized resonance for egg PC (cf. Fig. 1 B), again reflecting anisotropy changes. Similar results have also been obtained in our laboratories in studies with the opioid receptor (Salamon et al., 2000b; Alves et al., 2003), although with a smaller degree of anisotropy. It should be noted that for any of the bilayer compositions, before illumination of the sample neither the spectra nor the
spectral response to rhodopsin concentration changed over the pH range 5–8.5. This result indicates that in the dark the rhodopsin conformational state was not influenced by the buffer pH for any of the lipid systems used here. Changes in SPR spectra obtained with $p$-polarized light have also been observed in earlier studies involving rhodopsin performed in our laboratories (Salamon et al., 1994, 1999). Spectral analysis showed that these were caused by increases in the refractive index and in the thickness of the proteolipid system accompanying receptor incorporation, the latter associated with the protrusion of rhodopsin and lipid molecules from the surface of the lipid bilayer. The results presented in this article are consistent with these observations and extend them to include $s$-polarized resonances.

**PWR changes induced by light for rhodopsin incorporated into an egg PC bilayer**

Referring back to Fig. 1, panels A and B show that the exposure of rhodopsin to yellow light in an egg PC bilayer at pH 5 caused an increase in the resonance minimum angle in the PWR spectra for both polarizations. Similar results were obtained previously using SPR with $p$-polarized light (Salamon et al., 1994, 1999). There was also a slight increase in the amplitude of the PWR spectra for both polarizations (more pronounced in the case of the $s$-polarization). These spectral changes are due to an increase in mass density as well as to alterations in the mass distribution within the proteolipid membrane. Inasmuch as light irradiation neither added nor subtracted mass from the membrane, these changes were caused by rhodopsin conformational transitions which were coupled to infusion of lipid molecules from the Gibbs border to the bilayer. The spectral changes were quite anisotropic, with $p$-polarized resonance shifts (corresponding to the longer axis of the protein) larger than those with $s$-polarized light (22 mdeg vs. 14 mdeg). This extends the previous SPR results due to the ability of PWR to monitor $s$-polarized resonances, and constitutes a direct experimental confirmation of the photo-induced elongation of rhodopsin (i.e., increase in membrane thickness) seen in the earlier SPR experiments (Salamon et al., 1994, 1996).

When the same experiment was carried out in an egg PC bilayer at pH 7.5, as shown in Fig. 2, A and B, there were few or no changes induced in the PWR spectra upon light irradiation. Such a pH dependence of the light-induced PWR spectral changes correlates very well with previous studies using flash photolysis (Botelho et al., 2002), providing a strong indication that the PWR changes were due to formation of MII. The pH dependence of the light-induced PWR spectral change was further investigated for the egg PC recombinants by conducting experiments over an extended pH range. Fig. 3 shows the maximal resonance shifts (obtained under saturating light conditions) caused by rhodopsin irradiation as a function of pH. Fitting the data to the Henderson-Hasselbalch equation yielded a $pK_a$ value of 6.4 $\pm$ 0.05 for the acid-base equilibrium, which agrees very well with the analogous studies performed using flash photolysis (Gibson and Brown, 1993). This provides still further confirmation that MII formation was being observed in the PWR experiments.

**PWR changes induced by light for rhodopsin incorporated into a DOPC bilayer**

Fig. 4, A and B, shows the effects of incorporating rhodopsin into DOPC membranes at pH 5 on PWR spectra obtained with $p$- and $s$-polarization, respectively. Contrary to what
was observed in rhodopsin/egg PC films, for the DOPC recombinants there was a slight reduction in the PWR spectral amplitude for s-polarization upon rhodopsin incorporation. This new observation indicates that the bilayer lipid composition plays a major role in the structural changes induced in the membrane upon rhodopsin incorporation. Moreover, when rhodopsin was exposed to light the changes in the resonance positions in the PWR spectra were significantly smaller at pH 5 (8 mdeg and 5 mdeg for p- and s-polarized resonances, respectively) compared to the shifts observed in the case of egg PC films at the same pH (cf. Fig. 1). Taken together the findings demonstrate that the magnitude of the conformational changes induced in the proteolipid membrane upon MII formation are smaller for the DOPC bilayer. As with egg PC, there were no changes in the PWR spectra when the same experiment was performed at pH 7.5 (data not shown). This observation is consistent with the flash photolysis result that this pH is not favorable for MII formation in PC bilayers (Botelho et al., 2002).

PWR changes induced by light for rhodopsin incorporated into a DOPC/DOPE bilayer

Turning next to Fig. 5, A and B, it can be seen that incorporation of the receptor into DOPC/DOPE (25:75 mol %) recombinant films at pH 5 produced changes in the PWR spectra similar to those observed in the case of egg PC recombinants, with an increase in both the resonance positions and amplitudes. Again, light activation resulted in shifts to higher resonance angles for both p- and s-polarized resonances, which were even larger and more anisotropic than in the egg PC case (shifts of 29 mdeg and 18 mdeg were obtained for p- and s-polarization, respectively). In Fig. 6, we show the results for the same experiment at different pH values, yielding a pK_a value for the magnitude of the flash-induced PWR changes of 7.3 ± 0.13. This result is in good agreement with previous flash photolysis studies (Botelho et al., 2002), lending further support to the interpretation that the PWR changes reflect the MI-MII conformation change of photolyzed rhodopsin. Thus both PWR and flash photolysis support the view that the MI-MII equilibrium is shifted toward the MII state when phosphatidylethanolamine (PE) is added to the system.

We have also performed the same experiment using bilayers with different DOPC/DOPE molar ratios at pH 5 (Fig. 7), and have found that an increase in the content of DOPE leads to a larger limiting magnitude of the PWR spectral shifts observed upon rhodopsin activation. This is...

FIGURE 2 PWR spectra obtained upon light activation of rhodopsin incorporated into an egg PC lipid bilayer in 10 mM phosphate buffer, at pH 7.5, using p-polarized (A) and s-polarized (B) light excitation. All spectra were measured with 632.8 nm exciting light. (■) PWR spectra obtained after addition in the dark of an octylglucoside-containing buffer solution of rhodopsin to the aqueous compartment of the sample cell containing a preformed bilayer (final rhodopsin concentration in the cell sample compartment was ≈1 μM); (○) PWR spectra obtained upon saturating yellow light (λ > 500 nm) activation of rhodopsin. Solid lines represent the data; symbols are used to distinguish the two spectral curves. Note that the two sets of spectra superimpose on each other within the spectral resolution (1 mdeg) indicating that no rhodopsin photoactivation occurred.

FIGURE 3 Dependence on pH of the changes in the PWR resonance angular position observed in recombinant rhodopsin/egg PC films in 10 mM phosphate buffer upon saturating yellow light irradiation. Squares represent the results obtained with p-polarized light and triangles with s-polarized light. The solid curve through the data points for both polarizations represents the best fit to the Henderson-Hasselbalch equation with an apparent pK_a value of 6.4 ± 0.05.
a new observation that extends the flash photolysis results by directly demonstrating a greater extent of proteolipid membrane conformational change induced by MII formation. The greater limiting magnitude can be attributed to an increase in the contributions from the proteolipid restructuring to the stabilization of the MII state as the PE concentration is increased. It is also noteworthy that such a linear dependence on mol % DOPE is predicted by a previously proposed flexible surface model for membrane lipid-rhodopsin interaction (Botelho et al., 2002). In summary, we conclude that the data presented in Figs. 1–7 strongly support the contention that the changes in the PWR spectra observed here are due to the formation of the MII state of rhodopsin in the lipid bilayer upon light irradiation. New structural insights into the influence of the membrane environment on this process are thereby obtained.

PWR changes observed upon transducin interaction with the proteolipid system

Control experiments were first performed to measure the degree of \( G_t \) binding to a preformed DOPC/DOPE (25:75 mol %) lipid bilayer with no rhodopsin incorporated. Spectral shifts observed upon the interaction of \( G_t \) (final concentration 1 \( \mu \)M) with the lipid bilayer alone (Fig. 8) were very small (<10 mdeg for both \( p- \) and \( s- \)polarization)
compared to those obtained when rhodopsin was present. Hence one can conclude that binding of Gt to the solid-supported lipid bilayer in the absence of rhodopsin is very weak or nonexistent. We then added Gt to the PWR cell with a preformed DOPC/DOPE (25:75 mol %) lipid bilayer at pH 5 containing rhodopsin, and the PWR spectral changes were monitored. As seen in Fig. 9, A and B, addition of Gt to the proteolipid system caused a large increase in the angular position of the PWR spectra (≈60 mdeg for p- and ≈45 mdeg for s-polarized light), as well as a decrease in the signal amplitude. These changes are consistent with an increase in mass density and changes in mass distribution in the proteolipid system as a consequence of Gt binding to dark-adapted rhodopsin. It should also be noted that the PWR spectral changes were anisotropic (shifts larger for p- than s-polarized light), consistent with similar studies using the human δ-opioid receptor (hDOR) (Alves et al., 2003), suggesting an increase in bilayer thickness caused by Gt association with rhodopsin. The binding affinities of Gt to rhodopsin were obtained by measuring the shifts in the resonance minimum position of the PWR spectra at different Gt concentrations and fitting the data to a hyperbolic function.

Fig. 10, A and B, and Table 1 show that the affinity of Gt to rhodopsin in a DOPC/DOPE (25:75 mol %) bilayer was highly dependent on the activation state of the receptor, being much higher upon photoactivation (Kd for Rho* = 0.7 ± 0.1 nM) than in the dark state (Kd for Rho = 64 ± 11 nM). We attribute this to the activated receptor being in a more favorable conformational state for optimal Gt interaction, as expected from a wide variety of other experiments on GPCRs. By directly measuring Kd values, the experiments used in this study allow this expectation to be quantified. Other studies (Dunham and Farrens, 1999; Altenbach et al., 1999a,b, 2001a,b; Hubbell et al., 2003) have indicated that upon light
activation rhodopsin undergoes a conformational change that leads to reorientation of the transmembrane helices. Together with the results presented in this study one can conclude that the activation involves a protrusion of the protein from the lipid bilayer, thereby exposing regions of Gt recognition buried in the inactivated state of the receptor. When the same experiments were performed at pH 7.5, the Gt affinity either to dark-adapted or light-activated rhodopsin was comparable to that observed for dark-adapted rhodopsin at pH 5 (data not shown), further confirming that at pH 7.5 rhodopsin is largely shifted to the inactive MI state.

Experiments similar to those described above were also performed for rhodopsin incorporated into a DOPC lipid bilayer at pH 5 and 7.5. The results at pH 7.5 were similar to those obtained with the DOPC/DOPE bilayer, i.e., there was no difference in the affinity of Gt to Rho and Rho*, and the $K_d$ value was similar to that obtained at pH 5 for dark-adapted rhodopsin (data not shown). At pH 5 in the DOPC bilayer, the affinity of Gt to Rho* was increased from $68 \pm 9$ nM for dark-adapted rhodopsin to $18 \pm 3$ nM, but was still markedly less in comparison with the DOPC/DOPE bilayer (Table 1). This result is direct evidence that the lipid bilayer composition affects not only the receptor activation (Wiedmann et al., 1988; Gibson and Brown, 1993) but also the initial events of the signaling cascade in the visual process. One can attribute this to the fact that DOPC bilayers have a tendency to have zero spontaneous curvature (Brown, 1994), which is unfavorable for the conformational events occurring upon rhodopsin activation that lead to Gt binding.

PWR changes observed upon GTPγS interaction and light activation with rhodopsin/transducin

Referring back to Fig. 9, A and B, the interaction of GTPγS with the proteolipid system containing Rho* and Gt,
produced large anisotropic shifts in the PWR spectra to smaller resonance angles (−42 mdeg for \( p \)- and −30 mdeg for \( s \)-polarization). We attribute this to a decrease of mass in the proteolipid system as a consequence of the dissociation of the \( \alpha \)-subunit of transducin from the receptor and bilayer. Since the shift did not return to the spectrum to the original value, we assume that the entire heterotrimeric transducin molecule was not released from the membrane. Similar results were previously obtained for the interaction of GTP\( \gamma \)S with the hDOR-G protein complex (Alves et al., 2003). Based on recent studies involving the hDOR (Alves et al., 2004), in which it was found that the \( \beta \gamma \) dimer has higher affinity to the lipid bilayer (when in the absence of the \( \alpha \)-subunit) than the \( \alpha \)-subunit alone, we hypothesize that the \( \beta \gamma \) subunit remained bound to the proteolipid system after GTP\( \gamma \)S interaction. Similar changes in \( p \)-polarized spectra have been reported previously for rhodopsin using SPR (Heyse et al., 1997), and for PWR experiments performed using the hDOR (Alves et al., 2003). The affinity of GTP\( \gamma \)S for G\( \text{T-Rho}^* \) was determined to be \( \sim 700 \) nM, as shown in Fig. 11, and was independent of the lipid bilayer composition (Table 1). It is important to note that no PWR spectral changes were observed upon GTP\( \gamma \)S addition in the absence of prior rhodopsin photoactivation, i.e., G\( _i \) bound to Rho was not able to carry out the GTP/GDP exchange (data not shown). In this case, upon subsequent irradiation of the system a decrease in the amplitude of the PWR spectra and shifts to smaller resonance angles were observed. These results are a direct demonstration that the binding capability of G\( _i \) to the receptor and its activation are independent phenomena, and that the GTP/GDP exchange activity of G\( _i \) requires the presence of activated receptor. We conclude that the affinity of GTP\( \gamma \)S to G\( _i \) is dependent on the receptor activation state but independent of the lipid bilayer composition.

Again returning to Fig. 9, A and B, it should be noted that the light-driven conformational change of rhodopsin in the presence of G\( _i \) produced an increase in the resonance minimum position for both \( p \)- and \( s \)-polarized light in a DOPC/DOPE bilayer at pH 5. Although this was also true for a DOPC bilayer (data not shown), the magnitude of this conformational change was larger for the DOPC/DOPE bilayer (≈60 mdeg for \( p \)-polarization) than for the DOPC bilayer (≈40 mdeg for \( p \)-polarization), for reasons already discussed. It is also noteworthy that the limiting magnitude of the light-induced PWR change, regardless of the lipid bilayer composition, was smaller when G\( _i \) was present than when it was absent. Thus, in the case of the DOPC/DOPE (25:75 mol \%) bilayer, limiting PWR shifts of 27 mdeg for \( p \)-polarized light and 17 mdeg for \( s \)-polarized light were observed at pH 5 in the absence of G\( _i \) (Fig. 5), compared to a saturating magnitude of 14 mdeg for \( p \)-polarized light and 13 mdeg for \( s \)-polarization in the presence of 10 nM G\( _i \) (Fig. 9). This decrease in the limiting magnitude of the PWR spectral shifts indicates that the structural changes in the proteolipid system were different under these two conditions. Such a difference could originate from the lipid, the receptor, or the G\( _i \) or from any combination of these. However, in combination with the fact that the affinity for G\( _i \) was increased when rhodopsin was activated, these results provide a direct observation of the so-called high-affinity and low-affinity states of the receptor.

**CONCLUSIONS**

In these studies we have shown that PWR spectroscopy can be used to measure the effects of lipid bilayer composition and buffer pH on rhodopsin activation. The results correlate quite well with investigations previously conducted using flash photolysis techniques that monitor changes in the optical spectra occurring upon conversion of rhodopsin to the MII state (Botelho et al., 2002). Thus, both experiments show that photolysis of rhodopsin in the presence of PE compared to PC favors the activated MII state, with a shift of \( \sim 1 \) pH unit in the p\( K_a \) that describes the MI-MII transition.

### Table 1: Binding affinities between G\( _i \) and rhodopsin (dark-adapted and light-activated) and between G\( _i \) and GTP\( \gamma \)S in DOPC and DOPC/DOPE (25:75) bilayers at pH 5

<table>
<thead>
<tr>
<th>Bilayer composition</th>
<th>( K_d ) Dark-adapted</th>
<th>( K_d ) Light-activated</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC/DOPE (25:75)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G( _i )-Rhodopsin( nM )</td>
<td>68 ± 9</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>G( _i )-GTP( \gamma )S( nM )</td>
<td>*</td>
<td>748 ± 85</td>
</tr>
<tr>
<td>DOPC (25:75)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G( _i )-Rhodopsin( nM )</td>
<td>64 ± 11</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>G( _i )-GTP( \gamma )S( nM )</td>
<td>*</td>
<td>736 ± 94</td>
</tr>
</tbody>
</table>

The \( K_d \) values reported were obtained from plotting the resonance minimum position for the PWR spectra (\( Y \)) as a function of either G\( _i \) or GTP\( \gamma \)S concentration (\( X \)) and fitting the data to the following hyperbolic function that describes the 1:1 binding of a ligand to a receptor: \( Y = (B_{max} \times X)/(K_d + X) \). Here \( B_{max} \) represents the maximum bound concentration and \( K_d \) is the concentration of ligand required to reach half-maximal binding. The \( K_d \) value represents the average of those obtained for \( p \)- and \( s \)-polarized light.

*No PWR spectral changes were observed in this case.

**Figure 11** Binding curves for the interaction of GTP\( \gamma \)S with the light-activated rhodopsin-G\( _i \) complex in DOPC/DOPE (25:75 mol \%) films. Isotherms plot the shifts in the resonance angle minimum of the PWR spectra obtained after addition of aliquots of GTP\( \gamma \)S for \( p \)- (●) and \( s \)-polarized (▲) light. Solid curves correspond to hyperbolic fits to the data; dissociation constant values are given in Table 1.
Clearly PWR, being sensitive to conformational changes occurring in both the protein and the lipids upon receptor activation, rather than in the chromophore alone as in the flash photolysis measurements, provides additional structural information. Here we have observed that lipid bilayers with high contents of nonlamellar-forming lipids (DOPC versus DOPE), or a more diverse assortment of fatty acyl chains (egg PC versus DOPC), generate more anisotropic structural changes upon receptor incorporation. Additionally, these bilayer compositions favor the formation of the MII state of rhodopsin by allowing the bilayer to participate directly in the receptor conformational change, as evidenced by the larger and more anisotropic structural changes observed in the PWR spectra upon light activation. We attribute this to the increased propensity of these lipids to form the reverse hexagonal phase (HII), due to the relatively small headgroup in combination with bulky, less ordered acyl chains (Brown, 1994). The resulting balance of forces is described by the spontaneous curvature (Wiedmann et al., 1988; Gibson and Brown, 1993; Brown, 1994), which is the result of the lateral pressure profile across the bilayer. The negative spontaneous curvature favored by DOPE could facilitate insertion of the receptor into an appropriate lipid environment within the bilayer, as well as favor reorientation of the transmembrane helices that allow a protrusion of rhodopsin from the membrane in association with the conformational changes occurring upon receptor activation.

Decreased formation of MII in DOPC and egg PC compared to DOPC/DOPE mixtures has been previously observed by flash photolysis (Botelho et al., 2002), and has been interpreted as follows. For DOPC the spontaneous curvature is zero (Brown, 1994) and can match the curvature at the protein-lipid boundary in the MI state, rather than in the MII state, where the curvature is suggested to be negative. The consequent increase in curvature free energy associated with the MI-MII transition disfavors MII, as found experimentally by flash photolysis (Botelho et al., 2002) and now by PWR spectroscopy (vide supra). Moreover, enhanced MII formation due to DOPE has also been seen in the previous flash photolysis experiments (Botelho et al., 2002). The PWR experiments employed in this study contribute importantly to understanding the functional consequences of membrane restructuring due to the presence of the smaller PE headgroup, which has a tendency to promote reverse hexagonal HII phase formation due to its negative spontaneous curvature. According to this view, matching of the negative spontaneous curvature to the curvature at the protein-lipid hydrophobic boundary yields a reduction of the curvature free energy and promotes formation of the MII state.

The larger changes in the proteolipid membrane structure occurring upon receptor activation for PE- versus PC-containing bilayers (Fig. 7) has not been observed before. We attribute this to a contribution from restructuring of the bilayer lipids associated with the conformation changes of rhodopsin leading to receptor activation. By contrast, in the flash photolysis studies (Botelho et al., 2002) the maximal signal amplitude is the same regardless of lipid bilayer composition, i.e., the amount of MII formed is not changed. We suggest that the differences in the PWR spectral amplitudes for the different lipid compositions are due to the different spontaneous curvature properties of the lipids. In this view, both lipid and rhodopsin contribute to the PWR changes in the PE bilayer, whereas in the PC case, the smaller PWR spectral shifts are mainly due to the protein conformational change. Future studies involving the use of chromophore-labeled lipids could be used to provide further insight into this question (Salamon et al., 1999; Salamon and Tollin, 2001a).

We have also discovered that the affinity of G, with rhodopsin is greatly affected by the receptor activation state as well as by the lipid bilayer composition. Direct evidence has been obtained that G, is able to bind not only to light-activated but also to dark-adapted rhodopsin (Hamm et al., 1987), consistent with our previous SPR studies (Salamon et al., 1996). Other measurements of the affinity of G, to light-activated rhodopsin have produced Kd values comparable to ours (Bennett and Dupont, 1985; König et al., 1989; Mitchell et al., 2001). Moreover, we have observed similar changes in the affinity of G-proteins to different activation states of another GPCR, the human δ-opioid receptor (Alves et al., 2003). In this latter case the agonist-occupied receptor had a higher affinity for G-proteins than the antagonist- or inverse agonist-bound receptor. Since the dark-adapted state of rhodopsin corresponds to an inverse agonist-bound receptor, the result obtained in this study is consistent with this finding and suggests that GPCRs share common mechanisms of action.

Modulation of the coupling of G, to rhodopsin by the lipid bilayer composition has been also observed using spectrophotometry (Mitchell et al., 2001; Niu et al., 2001), in which chain unsaturation and cholesterol influenced both the affinity and the kinetics of the binding interaction. In these studies, we have found that nonlamellar-forming lipids such as DOPE, with a small headgroup in comparison to the bulky unsaturated acyl chains, favor this interaction relative to lamellar-forming lipids, i.e., those with larger headgroups such as DOPC. Furthermore, we have also observed that G, is only active, i.e., capable of GDP-GTP exchange, when bound to an active receptor, pointing to the idea that its affinity to rhodopsin and its activity are separate phenomena. This is also supported by the observation that GTPγS affinity to G, but not the affinity of G, to rhodopsin, was independent of the lipid bilayer composition. Thus we have shown that incorporation of DOPE into a PC-containing bilayer enhances the affinity of G, to light-activated rhodopsin, without influencing its affinity to dark-adapted rhodopsin or the ability of G, to undergo GTP-GDP exchange. The membrane environment in this instance specifically affects the agonist-induced conformational event that occurs during receptor function. This may have important implications for GPCR
biology. Taken together, the results presented here demonstrate the ability of PWR spectroscopy to monitor both mass density and conformation changes related to the lipid modulation of GPCR activation and signal transduction events. This will be especially important in applications to GPCRs for which flash photolysis techniques cannot be used. In addition, the observation that the lipid composition of the bilayer can enhance the ability of a GPCR to become activated and to bind a G-protein has important implications for the regulation of receptor activity by sorting into membrane microdomains (Simons and Toomre, 2000).

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