Plasmon-Waveguide Resonance Studies of Ligand Binding to the Human β_2-Adrenergic Receptor†

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ABSTRACT: Plasmon-waveguide resonance (PWR) spectroscopy is an optical technique that can be used to probe the molecular interactions occurring within anisotropic proteolipid membranes in real time without requiring molecular labeling. This method directly monitors mass density, conformation, and molecular orientation changes occurring in such systems and allows determination of protein−ligand binding constants and binding kinetics. In the present study, PWR has been used to monitor the incorporation of the human β_2-adrenergic receptor into a solid-supported egg phosphatidylcholine lipid bilayer and to follow the binding of full agonists (isoproterenol, epinephrine), a partial agonist (dobutamine), an antagonist (alprenolol), and an inverse agonist (ICI-118,551) to the receptor. The combination of differences in binding kinetics and the PWR spectral changes point to the occurrence of multiple conformations that are characteristic of the type of ligand, reflecting differences in the receptor structural states produced by the binding process. These results provide new evidence for the conformational heterogeneity of the liganded states formed by the β_2-adrenergic receptor.

G-protein coupled receptors (GPCRs)[1] represent a physiologically and pharmacologically important class of integral membrane proteins (*1*). These receptors respond to a diverse array of endogenous ligands such as hormones, neurotransmitters, and peptides, as well as to exogenous stimuli such as light, odor, and taste (*2*, *3*). Although GPCRs differ widely in their physiological roles, they share a common structural feature consisting of seven transmembrane helical segments connected by alternating intracellular and extracellular loops, with the amino terminus on the extracellular side and the carboxy terminus on the intracellular side. The β_2-adrenergic receptor (β_2-AR) is one of the most well characterized members of the rhodopsin family (family A) of GPCRs (*4*−*6*). This receptor is activated by endogenous catecholamines (epinephrine and norepinephrine) and is an important pharmaceutical target for pulmonary and cardiovascular diseases. Synthetic ligands that bind to the β_2-AR have a broad spectrum of functional properties including full agonists, partial agonists, neutral antagonists, and inverse agonists.

Molecular events in anisotropic systems, such as the activation of membrane receptors associated with ligand binding and subsequent structural changes, are difficult to characterize due to the complexity of the dynamic conformational changes that occur. Conventional fluorescence techniques and single molecule spectroscopy have been used to study the process of agonist activation in the β_2-AR (*7*−*9*). For the human δ-opioid receptor, plasmon-waveguide resonance (PWR) spectroscopy has been employed to probe the structural events following the binding of various classes of ligands (agonists, antagonists, and inverse agonists) in real time and without requiring any molecular labeling (*10*, *11*). These studies demonstrated distinguishable receptor conformational states following the binding of different classes of synthetic opiate ligands.

Figure 1 illustrates the application of PWR spectroscopy to the characterization of such systems. PWR spectral changes produced upon receptor incorporation into a preformed lipid bilayer deposited upon the surface of the plasmon resonator, as well as those produced by subsequent ligand binding, reflect changes in mass density and mass distribution, the latter caused by changes in structural anisotropy. Inasmuch as the molecular weights of the ligands used in this study are all quite similar (range ∼250−350), differences in the magnitudes of the angular shifts for *p*- and *s*-polarized resonances caused by the binding of various ligands must be due to differences in the proteolipid membrane structures thereby produced. It should also be noted that the kinetics of such spectral changes can reveal the presence of intermediate structural states. This approach has now been extended to the human β_2-AR, and new evidence for conformational and kinetic heterogeneity has been obtained.

MATERIALS AND METHODS

Preparation and Purification of the Receptor. DNA sequences encoding the human β_2-adrenergic receptor,

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[1] Abbreviations: PWR, plasmon-waveguide resonance; β_2-AR, β_2-adrenergic receptor; GPCR, G-protein coupled receptor; egg PC, egg phosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; mdeg, millidegree.

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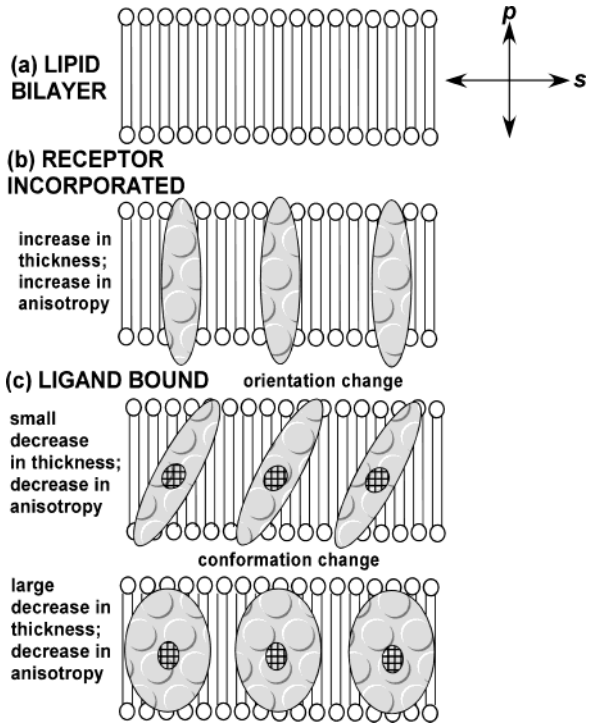

FIGURE 1: Application of PWR spectroscopy to membrane systems. Changes in properties (thickness, refractive indices, anisotropy) of a lipid bilayer (a) upon insertion of an integral membrane protein receptor (b) and upon binding of a ligand (c) that causes orientation or conformation changes in the receptor. Note that *p*- and *s*-polarized refractive indices can change differently depending upon the anisotropic nature of the structural changes, resulting in different patterns of spectral shifts and amplitude changes. Thickness and anisotropy changes are due to protein extending beyond the edges of the lipid bilayer and to changes in protein structural asymmetry, respectively.

epitope-tagged at the amino terminus with the cleavable influenza−hemagglutinin signal sequence followed by the "FLAG" epitope (IBI, New Haven, CT) and tagged at the carboxyl terminus with six histidines, were cloned into the baculovirus expression vector pVL1392 (Invitrogen, San Diego, CA) (*12*). This construct was cotransfected with linearized BaculoGold DNA into Sf9 insect cells using the BaculoGold transfection kit (Pharmingen, San Diego, CA). For purification, the cells were grown in 1000 mL cultures in SF 900 II medium (Gibco, Grand Island, NY) supplemented with 5% (v/v) fetal calf serum (Gemini, Calabasas, CA) and 0.1 mg/mL gentamicin (Boehringer Mannheim, Mannheim, Germany). Cells were infected with a 1:30−40 dilution of a high titer virus stock at a density of 3.5−5.5 × 10^6 cells/mL and harvested after 48 h by centrifugation (10 min at 5000*g*). The cell pellets are kept at −70 °C until used for purification. Receptor was purified using a two-step purification procedure. One to two pellets of Sf9 cells from 1000 mL infected cultures were resuspended in lysis buffer [10 mM Tris-HCl, pH 7.4, with 1 mM EDTA, 10 μg/mL benzamidine (Sigma), 10 μg/mL leupeptin (Boehringer Mannheim, Germany), and 0.2 mM phenylmethanesulfonyl fluoride (Sigma)]. Following centrifugation (20 min at 30000*g*), the lysed cells were resuspended in solubilization buffer [20 mM Tris-HCl, pH 7.4, with 1.0% *n*-dodecyl β-D-maltoside (NDM) (Anatrace), 100 mM NaCl, 10 μg/mL benzamidine, 10 μg/mL leupeptin, 0.2 mM phenylmethanesulfonyl fluoride, and 10 μM alprenolol (Sigma)], subjected to 25 strokes from a Dounce homogenizer, and then stirred for 2 h at 4 °C. The solubilized receptor was purified by chromatography using M1 Flag antibody affinity resin (Sigma) and alprenolol affinity chromatography as described (*12*). Approximately 5 nmol of pure protein (17 nmol/mg; theoretical is 20 nmol/mg) generally could be obtained from a 1000 mL culture.

Formation of a Solid-Supported Lipid Bilayer. Generation of a self-assembled solid-supported lipid membrane involves the spreading of 2 μL of lipid bilayer-forming solution [8 mg/mL egg phosphatidylcholine in butanol/squalene (93:7)] across a Teflon orifice which separates the silica waveguide surface from the aqueous phase (10 mM Tris-HCl containing 0.5 mM EDTA and 10 mM KCl at pH 7.3) in the PWR cell (*13*, *14*). The hydrophilic SiO_2 surface is covered with a thin layer of water of condensation (*15*, *16*) and attracts the polar groups of the lipid molecules with the hydrocarbon chains oriented toward the bulk lipid phase, which induces an initial orientation of the lipid molecules. The next step involves addition of aqueous buffer into the sample compartment of the PWR cell, which results in formation of a plateau−Gibbs border that anchors the membrane to the Teflon spacer. This border allows membrane flexibility wherein the lipid molecules can get displaced or recruited upon insertion of a membrane protein or as a result of ligand-induced protein conformational changes. In the present system, the average thickness of such a bilayer before protein incorporation is ∼5.5 nm, including the hydrated lipid headgroups (*17*). Lipid was purchased from Avanti Polar Lipids (Birmingham, AL). All experiments were done at ambient temperature.

PWR Spectroscopy. The PWR instruments used for these experiments were Aviv Alpha prototype and Beta version devices obtained from Proterion Corp. (Piscataway, NJ), both having a spectral resolution of 1 mdeg. The principle of PWR spectroscopy has been described extensively elsewhere (*13*, *18*, *19*). Briefly, resonant excitation by polarized light from a CW laser (λ = 632.8 or 543.5 nm) passes through a glass prism whose hypotenuse is coated with a metal film (Ag) and a SiO_2 waveguide overcoating under total internal reflection conditions. Excitation of surface plasmons creates an evanescent electromagnetic field localized at the outer surface of the waveguide layer, which is used to probe the optical properties of the molecules immobilized at this surface. Depending on the incident light polarization, resonances can be achieved in either the perpendicular (*p*) or the parallel (*s*) direction relative to the bilayer surface. This allows the characterization of anisotropic systems, such as integral membrane proteins incorporated in the lipid bilayer (*10*, *14*, *17*, *20*). PWR has an advantage over conventional SPR studies, which can use only *p*-polarized excitation and therefore cannot provide information such as anisotropy changes due to structural transitions.

A PWR spectrum is obtained by plotting the reflected light intensity as a function of the incident angle and can be described by three parameters: the angular position, the width, and the depth. The spectrum is determined by the refractive index (*n*), extinction coefficient at the excitation wavelength (*k*), and the sample thickness (*t*). Although these optical parameters can be obtained by fitting the PWR spectra to Maxwell's equations applied to thin film assemblies (*18*, *19*, *21*), the present report will be limited to the qualitative aspects of the protein−ligand interactions.

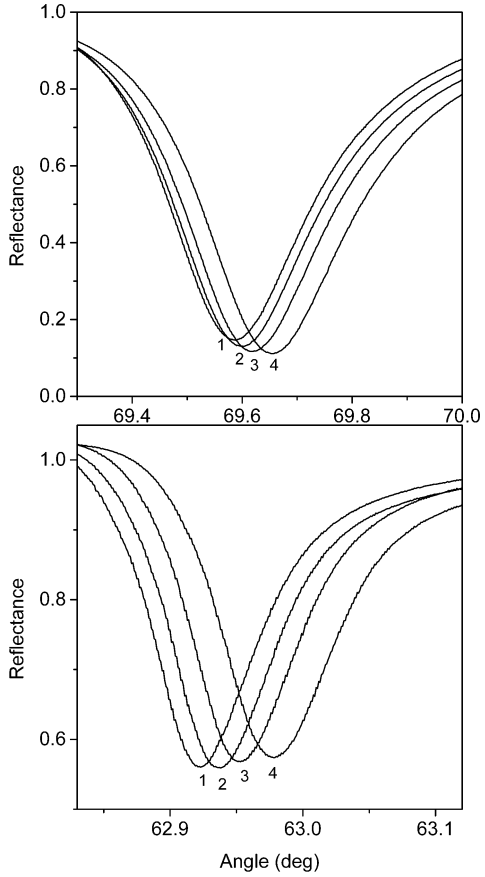

FIGURE 2: β_2-Adrenergic receptor incorporation into a solid-supported lipid bilayer. PWR spectra of receptor molecules dissolved in an octyl glucoside containing buffer and incorporated into an egg phosphatidylcholine bilayer by detergent dilution are shown for both *s*- (upper panel) and *p*- (lower panel) polarization. Final receptor concentration in the bulk solution is 0 (curve 1), 2 nM (curve 2), 6 nM (curve 3), and 14 nM (curve 4). Aqueous buffer in the sample cell was 10 mM Tris-HCl containing 0.5 mM EDTA and 10 mM KCl at pH 7.3.

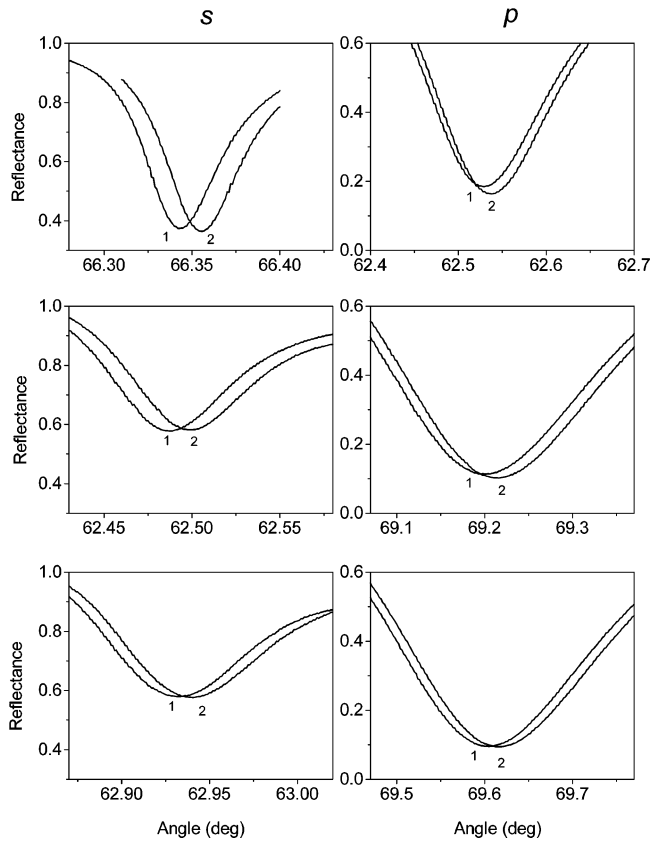

FIGURE 3: Agonist-induced changes in PWR spectra. PWR spectra of β_2-adrenergic receptor incorporated into an egg PC lipid bilayer before and after agonist ligand addition. Buffer conditions are as in Figure 2. Magnitudes of spectral shifts are given in Table 1. Upper panel: Spectrum obtained before (curve 1) and after (curve 2) addition of the full agonist (−)isoproterenol (bulk concentration = 4 μM) to the aqueous sample cell for both *s*- (left panel) and *p*- (right panel) polarization after equilibration. Middle panel: Spectrum obtained before (curve 1) and after (curve 2) addition of the full agonist epinephrine (bulk concentration = 5 μM) for *s*- (left panel) and *p*- (right panel) polarization after equilibration. Bottom panel: Spectrum obtained before (curve 1) and after (curve 2) addition of the partial agonist dobutamine (bulk concentration = 3 μM) for *s*- (left panel) and *p*- (right panel) polarization after equilibration.

RESULTS

β_2-Adrenergic Receptor Incorporation into a Solid-Supported Lipid Bilayer. β_2-AR molecules were incorporated into a preformed lipid bilayer deposited on the hydrophilic silica surface (see Materials and Methods) by addition of small aliquots of a concentrated solution of the protein dissolved in 30 mM octyl glucoside detergent-containing buffer to the aqueous volume of the sample cell. This dilutes the detergent to a final concentration below its critical micelle concentration (25 mM for octyl glucoside), which results in a spontaneous transfer of the receptor molecules from the micelles into the bilayer. Insertion of a membrane-spanning protein such as a receptor results in the hydrophobic portion of the protein localizing within the bilayer interior and the peripheral portions of the protein extending outside the membrane on each side of the bilayer to form extramembrane protein layers in contact with the aqueous medium (*17*, *20*). This produces an increase in the thickness of the bilayer as well as refractive index changes, which result in PWR spectral changes. In these experiments, the *k* parameter is due largely to scattering effects, inasmuch as the PWR excitation wavelengths are not absorbed by any of the sample components. Figure 2 shows PWR spectra obtained for *p*- and *s*-polarized exciting light for a lipid membrane prior to and after incorporation of increasing amounts of solubilized receptor. Any alprenolol that was bound to the receptor for stabilization during the purification procedure was removed after receptor incorporation by washing the proteolipid membrane with buffer solution before addition of aliquots of a given ligand. This process could be monitored by changes in the PWR spectra (not shown). Note that the depth and angular position of the PWR spectrum are both influenced by the incorporation of the integral membrane receptor protein. This is a consequence of both mass density and structural changes in the membrane. That these structural changes are anisotropic is shown by the fact that the directions of the PWR amplitude changes are opposite in sign for *p*- and *s*-polarizations. This is a consequence of the asymmetric (i.e., cylindrical) structure of the inserted receptor, which is uniaxially oriented within the lipid bilayer (cf. Figure 1).

PWR Spectral Changes Occurring upon Binding Different Ligands to the Receptor. Changes in PWR spectra caused by receptor−ligand interactions are shown in Figures 3 and 4. These reflect alterations in the *n* and *t* values of the

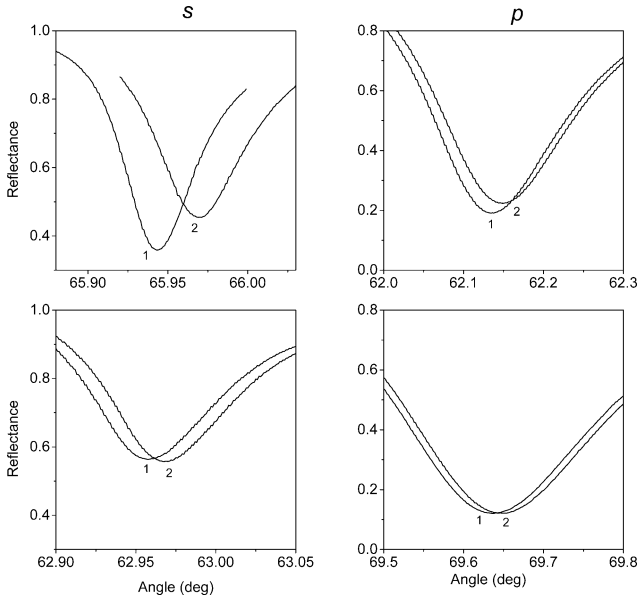

FIGURE 4: Effect of inverse agonist and antagonist binding on PWR spectra. PWR spectra of β_2-adrenergic receptor incorporated into an egg PC lipid bilayer before and after inverse agonist and neutral antagonist addition. Buffer conditions are as in Figure 2. Magnitudes of spectral shifts are given in Table 1. Upper panel: Spectrum obtained before (curve 1) and after (curve 2) addition of the inverse agonist ICI-118,551 (bulk concentration = 2.5 nM) for *s*- (left panel) and *p*- (right panel) polarization after equilibration. Lower panel: Spectrum obtained before (curve 1) and after (curve 2) addition of the neutral antagonist alprenolol (bulk concentration = 2.5 nM) for *s*- (left panel) and *p*- (right panel) polarization after equilibration.

Table 1: Magnitudes of Spectral Shifts for Ligand Binding to the β_2-AR[a]

ligand	*s*-shifts (mdeg)	*p*-shifts (mdeg)
(−)isoproterenol	12 ± 1	9 ± 1
epinephrine	12 ± 1	17 ± 2
dobutamine	9 ± 1	13 ± 1
ICI-118,551	26 ± 1	14 ± 2
alprenolol	9 ± 1	13 ± 1

[a] Data from Figures 3 and 4.

proteolipid membrane resulting from mass increases and/or changes in conformation (*10*, *11*, *14*, *17*). In what follows we summarize the qualitative aspects of the spectral changes associated with agonist, partial agonist, inverse agonist, and neutral antagonist interactions with the membrane-bound β_2-AR.

As can be seen from the spectra in Figures 3 and 4, all of the ligands used here cause shifts in the PWR spectra to larger angles upon binding to the receptor. Differences in the magnitudes of *s*- and *p*-polarized resonance shifts (see Table 1) reflect differences in the anisotropic nature of the structural perturbations. As pointed out above, all of these ligands have very similar masses, so that differences in spectral behavior are due mainly to differences in structural changes in the proteolipid membrane induced by ligand binding. Furthermore, differences in the kinetic behavior of the structural responses are seen between the various types of ligands included in this study (see below).

Concentration Dependence of Ligand Binding to the Incorporated Receptor. Addition of successive aliquots of a ligand solution to the sample cell resulted in changes in the

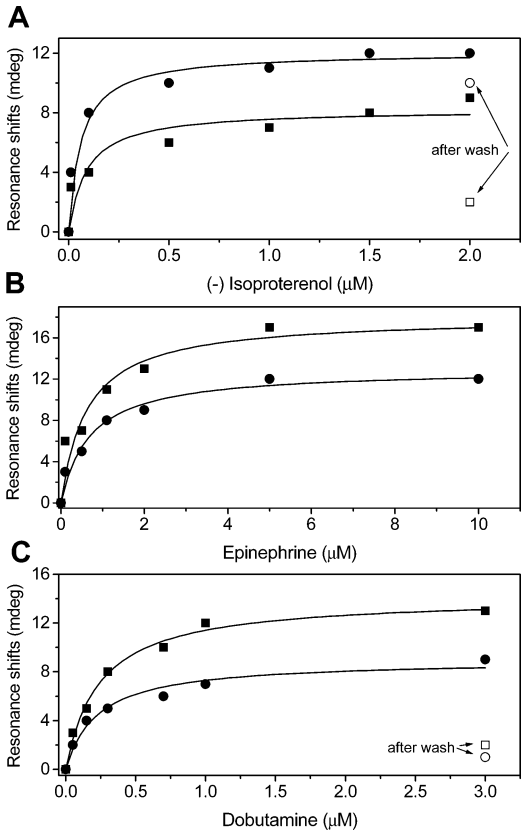

FIGURE 5: Binding curves for agonist interaction with β_2-adrenergic receptor. PWR spectral shifts obtained after ligand addition to β_2-adrenergic receptor incorporated into an egg PC lipid bilayer for *s*-polarization (closed circles) and *p*-polarization (closed squares). Buffer conditions are as in Figure 2. Panel A: Addition of aliquots of the full agonist (−)isoproterenol to the aqueous compartment. The solid lines indicate hyperbolic fits to the data with the K_D values given in Table 2. The effect of displacing the bound ligand at 2 μM concentration from the receptor by washing with buffer (open symbols) is shown. Panel B: Addition of aliquots of the full agonist epinephrine to the aqueous compartment. The solid lines indicate hyperbolic fits to the data with the K_D values given in Table 2. Panel C: Addition of aliquots of the partial agonist dobutamine to the aqueous compartment. The solid lines indicate hyperbolic fits to the data with the K_D values given in Table 2. Washing with buffer after ligand saturation (open symbols) is shown.

resonance spectra caused by binding to the incorporated β_2-AR. Ligand binding was observed to be dose-dependent and stereospecific. The concentration dependence can be illustrated by plotting the resonance position shifts as a function of the bulk concentration of the added ligand (Figures 5 and 6). The magnitudes of the shifts produced by ligand binding under saturation conditions (cf. Figures 3 and 4) are given in Table 1. Binding constants obtained by fitting the concentration dependence data to a hyperbolic function are given in Table 2 and are compared with literature values obtained using radiolabeling methods. In most cases (dobutamine is an apparent exception), the agreement is satisfactory considering the large ranges observed. Control experiments in which these ligands were added to a preformed lipid membrane in the absence of the receptor showed no measurable effects on PWR spectra, indicating that nonspecific binding was not observed in these experiments. The effects on the PWR spectra of the different classes of ligands used in this study, i.e., the full agonists (−)isoproterenol and epinephrine, the partial agonist dobutamine, the antagonist

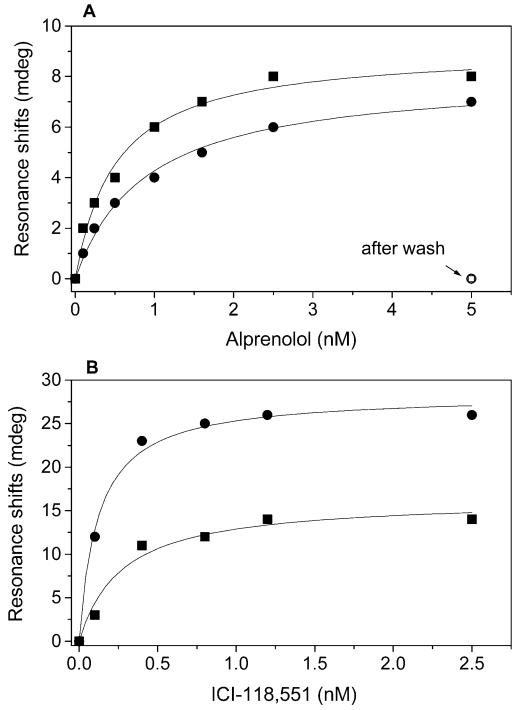

FIGURE 6: Binding curves for inverse agonist and antagonist interaction with β_2-adrenergic receptor. PWR spectral shifts obtained after ligand addition to the β_2-adrenergic receptor incorporated into an egg PC lipid bilayer for *s*-polarization (closed circles) and *p*-polarization **(**closed squares**)**. Buffer conditions are as in Figure 2. Panel A: Addition of aliquots of the neutral antagonist alprenolol to the aqueous compartment. The solid lines indicate hyperbolic fits to the data with the K_D values given in Table 2. Washing with buffer after ligand saturation (open symbols) is shown. Panel B: Addition of aliquots of the inverse agonist ICI-118,551 to the aqueous compartment. The solid lines indicate hyperbolic fits to the data with the K_D values given in Table 2.

Table 2: Equilibrium Dissociation Constants Obtained for Ligand Binding to the Incorporated β_2-AR

ligand	K_D		binding affinity (K_I)[a]
	s-polarization	*p*-polarization	
(−)isoproterenol	0.15 ± 0.01 μM	0.30 ± 0.02 μM	0.07−0.3 μM
epinephrine	0.7 ± 0.3 μM	0.6 ± 0.2 μM	0.4−4.3 μM
dobutamine	0.22 ± 0.05 μM	0.24 ± 0.03 μM	1.4−2.3 μM
ICI-118,551	0.12 ± 0.01 nM	0.27 ± 0.08 nM	0.2−1.2 nM
alprenolol	0.9 ± 0.1 nM	0.5 ± 0.1 nM	0.11−3 nM

[a] See refs *29*−*34*.

alprenolol, and the inverse agonist ICI-118,551, are summarized below.

Full Agonists Isoproterenol and Epinephrine. The concentration dependence of PWR spectral shifts (*s*- and *p*-polarization) upon addition of aliquots of the strong full agonist (−)isoproterenol to the aqueous compartment is plotted in Figure 5A. Note that the magnitude of the shifts for *s*-polarization was greater than for *p*-polarization, indicative of anisotropy changes in the proteolipid membrane (cf. Figure 1). These can be ascribed to changes in the molecular orientation of lipid and protein molecules within the membrane as well as to protein conformation changes. Ligand binding was saturable and could be fit with a hyperbolic function. The binding constants derived from these data were consistent with those obtained from conventional competition binding assays (Table 2), indicating that native behavior was observed.

As shown in Figure 5A, the spectral effect of displacing the bound ligand from the receptor by washing with buffer was different for *s*- and *p*-polarization (open circles). Thus, the observed angular position obtained after washing corresponded to almost the unliganded receptor value for the *p*-polarized resonance, whereas the *s*-polarized resonance was only partially shifted back to its original value. This implies that although the drug could be washed out, the structural state produced by the bound ligand did not revert to its original conformation, thereby leaving the receptor in an altered structure. The agonist-induced spectral changes could also be partially reversed by addition of the high-affinity neutral antagonist alprenolol after isoproterenol binding, without prior washing out of the agonist, consistent with what has been observed in previous studies (*8*). It is also possible that the partial structural reversibility was due to interference by the resonator−bilayer interface with the conformational change associated with ligand removal. We consider this to be unlikely since, as will be shown below, full reversibility was obtained with other ligands. Binding of the physiologically less active stereoisomer (+)isoproterenol to the receptor produced only insignificant spectral changes when added over the same concentration range as the (−) isomer (data not shown). This demonstrates the stereospecificity of the binding process.

Addition of the full agonist epinephrine to the incorporated receptor resulted in an increase in angular position for *p*-polarization that was larger than that for *s*-polarization (Figure 5B), which is different from the shifts obtained for the full agonist (−)isoproterenol (Table 1). This also indicates a change in anisotropy in the proteolipid membrane as a result of drug interaction, although the structural change was different from that produced by isoproterenol. The binding constant obtained from these data is given in Table 2.

Partial Agonist Dobutamine. The weak partial agonist dobutamine behaved similarly to the full agonist epinephrine, in that there was a larger shift for the *p*-polarized resonance than for *s*-polarization (Figure 5C). The binding constant values obtained from a hyperbolic fit to the data are given in Table 2. After saturation of the receptor with this ligand, washing with buffer resulted in reversal of the angular positions for both *s*- and *p*-polarized resonances to almost the unliganded angular position (Figure 5C), indicating virtually complete return to the original receptor conformation.

To summarize, among the agonists, there is a distinct difference in the pattern of angular shifts upon ligand binding, even between the full agonists (−)isoproterenol and epinephrine, as well as with the partial agonist dobutamine. This is indicative of differences in the anisotropy changes induced in the proteolipid membrane by these ligands, reflecting differences in receptor recognition of the specific ligand molecules and conformational heterogeneity in the different receptor states produced.

Neutral Antagonist Alprenolol. Addition of increasing concentrations of the neutral antagonist alprenolol resulted in larger shifts for the *p*-polarized resonance compared to *s*-polarization (Figure 6A). This is similar to the patterns observed with the partial agonist dobutamine, suggesting that similar conformational states of the proteolipid membrane were produced. The binding affinity values are presented in Table 2. The bound ligand could be washed off by buffer,

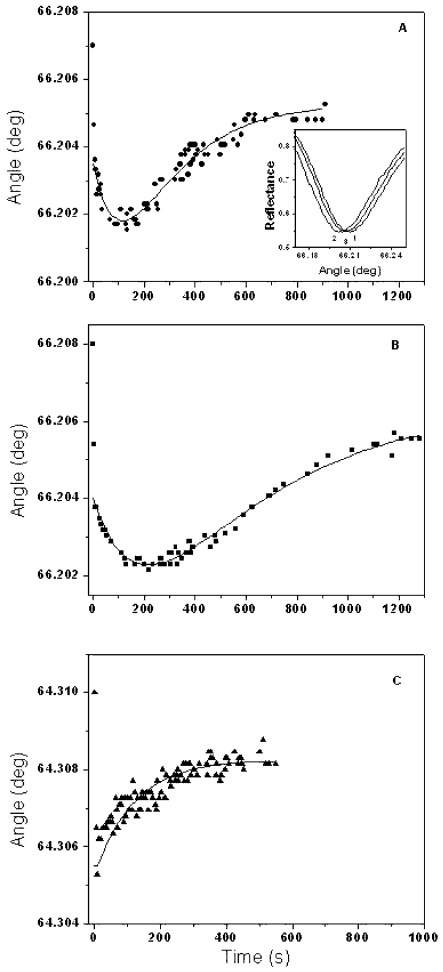

FIGURE 7: Kinetics of spectral shifts for agonist binding at a saturating concentration to the receptor. Buffer conditions are as in Figure 2. Panel A: Time course of spectral shifts for *s*-polarization for the full agonist (−)isoproterenol (bulk concentration = 10 μM). The solid line is a two-exponential fit to the data with $t_{1/2}$ values given in Table 3. Inset: Corresponding time-resolved PWR spectra using *s*-polarized light. Curve 1 was obtained before receptor incorporation; curve 2 represents the initial fast shift to smaller angles after addition of agonist; curve 3 was obtained after equilibrium. Panel B: Time course of spectral shifts for *s*-polarization for the full agonist epinephrine (bulk concentration = 10 μM). The solid line is a two-exponential fit to the slower phases of the data with $t_{1/2}$ values given in Table 3. Panel C: Time course of spectral shifts for *s*-polarization for the partial agonist dobutamine (bulk concentration = 10 μM). The solid line is a single exponential fit to the data with $t_{1/2}$ values given in Table 3.

resulting in reversion of the angular positions to their original values with both *s*- and *p*-polarized excitation light. This also suggests structural reversal.

Inverse Agonist ICI-118,551. The addition of aliquots of the inverse agonist ICI-118,551 to the membrane-bound β_2-AR produced significantly larger positive shifts for *s*-polarized resonances relative to *p*-polarization than were observed for any of the other ligands (Table 1, Figure 6B). This indicates a larger degree of anisotropy in the structural changes in the proteolipid membrane produced by binding this ligand to the receptor. Also note the larger decrease in the spectral amplitude of the *s*-polarized resonance compared to the *p*-polarized resonance observed for this ligand relative to the others (cf. Figure 4), again reflecting the formation of a different structural state. The binding data could be fit with a hyperbolic function with binding constants as given in Table 2. Bound drug could be washed from the receptor by buffer, which resulted in almost complete return of the spectrum to the unliganded angular position (not shown), again indicating structural reversibility.

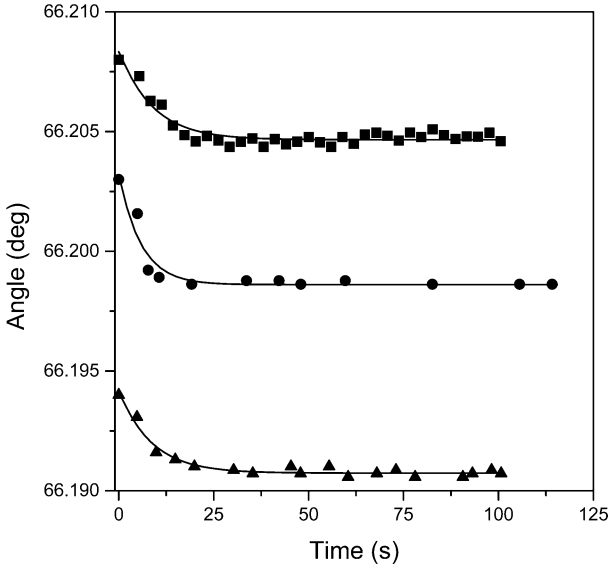

FIGURE 8: Kinetics of reversal of agonist-induced spectral shifts. Curves represent changes occurring upon subsequent addition of the high-affinity neutral antagonist alprenolol (bulk concentration = 10 μM) using *s*-polarization: isoproterenol (closed squares), epinephrine (closed circles), and dobutamine (closed triangles). The curves are shifted for comparison. The solid lines are single exponential fits to the data with $t_{1/2}$ values given in Table 3.

Kinetics of PWR Spectral Changes Induced by Ligand Binding. Upon addition to incorporated receptor at saturating concentrations, all classes of ligands showed distinctly different kinetic behavior for the spectral changes occurring upon binding to the β_2-AR. Kinetic measurements were done both at 10 μM ligand concentrations and 100 μM concentrations for agonists and at 10 and 100 nM concentrations for the antagonist and inverse agonist to ensure that the ligands completely occupied the receptor binding site in all cases. Both low and high ligand concentrations yielded similar kinetic behavior, and only data obtained with the lower concentrations will be shown below. It should be pointed out that the kinetic plots shown here represent only the initial stages of the time course of the spectral shifts. In all cases, there was an additional slower component that resulted in the final equilibrium shift values displayed in Figures 3−6. This is not included in Figures 7−9.

The time course of the spectral shifts for *s*-polarization for the full agonist (−)isoproterenol is plotted in Figure 7A. An initial very rapid shift to lower incident angles occurred upon ligand binding (within the time resolution of the instrument), followed by a subsequent slow decrease in resonance angle position and a still slower resonance angle increase, with equilibrium being reached in about 15 min. This multiphasic kinetic pattern clearly indicates the occurrence of at least three intermediate states during the binding process. Although all of these can reflect different conformations of the receptor−lipid system, the possibility also exists that one of the slower phases was due to the ligand moving across the bilayer and binding to receptors that were oriented with their ligand binding site facing the resonator surface. This requires further study. An exponential fit to the slower

Table 3: Kinetic Rate Constants and Half-Times Obtained for Ligand Binding to Incorporated β_2-AR

	$t_{1(1/2)}$, s	k_1, s^{-1}	$t_{2(1/2)}$, s	k_2, s^{-1}	$t_{3(1/2)}$, s	k_3, s^{-1}
ligand						
(−)isoproterenol	<10	<0.07	129 ± 11	5.4×10^{-3}	178 ± 16	3.9×10^{-3}
epinephrine	<10	<0.07	202 ± 14	3.4×10^{-3}	470 ± 22	1.5×10^{-3}
dobutamine	<10	<0.07	149 ± 43	4.6×10^{-3}		
ICI-118,551	<5	<0.2				
alprenolol	18 ± 2.1	0.04				
agonist reversal by alprenolol addition						
(−)isoproterenol	8.9 ± 1.01	0.078				
epinephrine	5.8 ± 1.0	0.12				
dobutamine	8.6 ± 0.9	0.08				

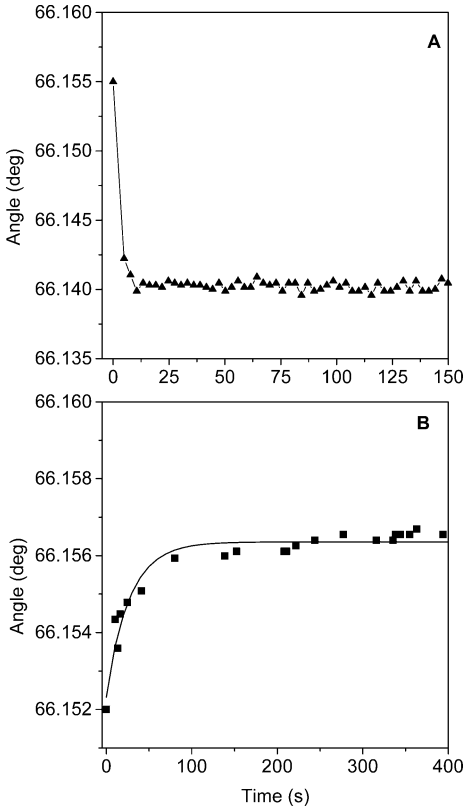

FIGURE 9: Kinetics of spectral shifts for inverse agonist and antagonist binding at saturating concentration to the receptor. Buffer conditions are as in Figure 2. Panel A: Time course of spectral shifts for *s*-polarization for the inverse agonist ICI-118,551 (bulk concentration = 10 nM). The solid line connects the data points and does not represent a fit. Panel B: Time course of spectral shifts for *s*-polarization for saturating concentrations of the antagonist alprenolol. A single exponential fit to the data is shown as a solid line with a $t_{1/2}$ as given in Table 3.

data phases yielded half-times of 129 and 178 s for these two processes (Table 3). Multiphasic agonist binding kinetics have also been observed with the δ-opioid receptor, the neurokinin receptor, the α_{2A}-adrenoceptor, and a minimized insulin receptor (*10*, *22*−*24*). In the case of the opioid receptor, we have suggested that the slow multiphasic kinetics were a consequence of structural changes induced in the bilayer by the receptor conformational change (*10*).

Figure 7B shows that binding of the agonist epinephrine followed a similar time course for *s*-polarization to that observed with isoproterenol, with a biphasic shift to lower angles followed by a slower change to larger angles. A two-exponential fit to the slower portions of the data yielded half-times of 202 and 470 s, respectively (Table 3). Binding of the weak partial agonist dobutamine (Figure 7C) appeared biphasic, with a very rapid decrease (within the time resolution of the instrument) followed by a slow increase with a half-time of 149 s.

The spectral changes induced by agonist and partial agonist binding were partially reversible by addition of the high-affinity antagonist alprenolol. The kinetics of this reversal are shown in Figure 8, and the corresponding half-times are reported in Table 3. As is evident, the time course of reversal was much faster than for the agonist-induced changes, was monophasic, and occurred with similar half-times for all three ligands. This suggests that the rate-limiting step may be the binding of the antagonist to the agonist-occupied receptor; this is consistent with the rapid structural changes induced by antagonist binding as will be shown below.

In sharp contrast to the results for agonists, both the direction of the resonance shifts and the time course of the shifts for the inverse agonist ICI-118,551 depended upon the concentration range used. At lower concentrations (<2.5 nM) the shifts occurred in the positive direction (Figure 6B) with a half-time for *s*-polarization of 600 s (not shown). For this ligand, the time required for *p*-polarized resonance changes to reach equilibrium was approximately two times that for the *s*-polarized resonance (not shown). This suggests that the time course of the structural changes was also anisotropic; i.e., the changes occurring in the membrane plane were slower than those occurring perpendicular to the plane of the membrane. Even more striking, however, when an aliquot of the ligand was added at higher concentrations (>10 nM), the shifts occurred in the opposite direction and with much faster kinetics. This is shown in Figure 9A. It was not possible to obtain an accurate half-time for this latter process inasmuch as most of the change occurred within the time resolution of the experiment. The explanation for this unusual behavior is not clear at present, and further studies are required. However, it is clear that this ligand exhibited unique binding kinetics.

Addition of the antagonist alprenolol at both low and saturating concentrations resulted in a relatively rapid monophasic time course of binding to the receptor (Figure 9B; half-time given in Table 3). This is similar to results obtained with the δ-opioid receptor (*10*).

DISCUSSION

The spectral and kinetic results presented above have provided direct evidence that different receptor structural states of the β_2-AR are produced by the binding of different ligand types. In the case of the human δ-opioid receptor,

the addition of a given class of ligand has also been shown to produce PWR spectral changes that are characteristic of the ligand type (*10*, *11*). For this receptor, agonists and inverse agonists caused shifts for both *p*- and *s*-polarized resonances to larger incident angles, whereas antagonists caused shifts for *p*-polarized resonances to larger angles and *s*-polarized resonances to smaller incident angles. In addition, it was shown that the kinetics of the resonance changes were different for agonists and antagonists. Although the details of the spectral shift results reported here for the β_2-AR system are rather different from those observed with the opioid receptor, the kinetic differences observed for agonists and antagonists are similar and the same general conclusion has been reached, i.e., that different receptor structural states are produced by different ligand types. Thus, this may be a general characteristic of the GPCR superfamily, although more study is required to support such a generalization.

These studies can be compared with spectroscopic studies in which conformational changes in the β_2-AR were detected by a fluorophore covalently bound to Cys265 at the cytoplasmic end of transmembrane segment 6 adjacent to a G-protein coupling domain (*8*, *25*). Binding of agonists and partial agonists resulted in changes in fluorescence intensity that correlated with the efficacy of the agonist (partial agonists producing smaller fluorescence intensity changes than full agonists). In contrast, no detectable changes in fluorescence intensity were observed on binding of antagonists or inverse agonists (*8*). In the present studies, shifts in *p*-polarized and *s*-polarized resonances were detected upon binding to all classes of ligands. It should be noted that these shifts correspond to the equilibrium structural states of the entire proteolipid membrane attained after ligand binding, whereas the fluorescence measurements largely reflect local changes within the receptor protein. Thus, it is certainly possible for differences to occur in these two types of measurements. Also, *in vivo* the presence of other proteins (e.g., G-proteins), as well as differences in membrane composition, may stabilize additional conformational states. As a consequence, the structural changes reflected in the PWR spectra need not be predictive of ligand efficacy.

It is also noteworthy that significant differences were observed in the PWR spectra of isoproterenol and epinephrine, both full agonists that differ only in the amine substituent (methyl for epinephrine, isopropyl for isoproterenol). Thus, while the structural difference between epinephrine and isoproterenol has minimal functional consequences, these agonists produced structural differences in the β_2-AR-containing membrane that were readily detected by PWR spectroscopy.

Fluorescence lifetime studies of β_2-AR labeled at Cys265 with fluorescein showed that the receptor is a conformationally flexible molecule. While binding of the antagonist alprenolol did not result in a change in fluorescence intensity or fluorescence lifetime, there was a narrowing in the distribution of fluorescence lifetimes, suggesting a reduction in molecular flexibility (*25*). In agreement with the present PWR studies, lifetime experiments demonstrated that agonists and partial agonists stabilize distinct conformational states (*25*). These results are consistent with theoretical considerations that predict GPCRs can adopt numerous conformations with different functional properties (*26*−*28*).

As shown above, the kinetics of PWR spectral changes for agonists and partial agonists were multiphasic, while those for antagonists were monophasic. These results suggest that the process of agonist binding and receptor activation occurs through discrete conformational intermediates. This interpretation is consistent with fluorescence lifetime experiments on the β_2-AR (*25*). Thus, a single lifetime distribution was observed in the unliganded receptor and antagonist-bound receptor, whereas two lifetimes were observed in the receptor bound to saturating concentrations of agonists and partial agonists (*25*). Moreover, recent fluorescence experiments have detected a biphasic conformational response to agonists. For isoproterenol, the rapid component had a $t_{1/2}$ of ∼4 s and the slow component had a $t_{1/2}$ of ∼150 s, and for epinephrine the values were ∼5 and ∼165 s (Swaminath et al., in press). This suggests that the receptor activation by agonists occurs through one or more kinetically distinguishable intermediate states. These observations are consistent with the present PWR experiments. Also consistent with the present results are studies of the β_2-AR receptor using single molecule spectroscopy (*9*) in which at least three states were observed for isoproterenol binding and were attributed to stabilization of conformational substates.

CONCLUSIONS

These results have shown that PWR spectroscopy can probe real time interactions occurring during the binding of various ligands to a proteolipid system and can thereby provide information concerning the structural changes of anisotropic integral membrane proteins such as GPCRs. The observations reported here are consistent with those obtained using other optical spectroscopic methods, such as fluorescence, and provide additional evidence for multiple conformational states resulting from ligand binding to the β_2-AR. However, the fact that PWR reports on changes occurring in both the protein and lipid components makes it complementary to information provided by these other techniques. Extensions of these studies could include the effect of different lipid bilayer compositions upon ligand binding to the receptor, as well as the structural and kinetic behavior of ligand-induced G-protein binding. Such work is presently underway.

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