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Different Structural States of the Proteolipid Membrane Are Produced by Ligand Binding to the Human δ-Opioid Receptor as Shown by Plasmon-Waveguide Resonance Spectroscopy

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ABSTRACT

Understanding structure-function relationships and mechanisms of signal transduction in G-protein-coupled receptors (GPCRs) is becoming increasingly important, both as a fundamental problem in membrane biology and as a consequence of their central role as pharmacological targets. Their integral membrane nature and rather low natural abundance present many challenging problems. Using a recently developed technique, plasmon-waveguide resonance (PWR) spectroscopy, we investigated the structural changes accompanying the binding of ligands to the human δ-opioid receptor (hDOR) immobilized in a solid-supported lipid bilayer. This highly sensitive technique can directly monitor changes in mass density, conformation, and orientation occurring in such thin proteolipid films. Without requiring labeling protocols, PWR allows the direct determination of binding constants in a system very close to the receptor's natural environment. In the present study, conformational changes of a proteolipid membrane containing the hDOR were investigated upon binding of a variety of peptide and nonpeptide agonists, partial agonists, antagonists, and inverse agonists. Distinctly different structural states of the membrane were observed upon binding of each of these classes of ligands, reflecting different receptor conformational states, and the formation of each state was characterized by different kinetic properties. Binding constants, obtained by quantifying the extent of conformational change as a function of the amount of ligand bound, were in good agreement with published values determined by radiolabeling methods. The results provide new insights into ligand-induced GPCR functioning and illustrate a powerful new protocol for drug development.

It is estimated that more than a thousand different G-protein-coupled receptors (GPCRs) exist in mammals, thereby constituting one of the largest protein families in nature (Watson and Arkinstall, 1994). Opioid receptors belong to this superfamily. They mediate the effects of opioid alkaloids and peptides on many important physiological functions, including stress, nociception, mood, reward, locomotor activities, respiratory functions, neurotransmitter release, and immune responses (Pasternack, 1988). Opioid receptors are classified into three different types, μ, δ, and κ, distinguished on the basis of extensive pharmacological, physiological, and behavioral studies (Gilbert and Martin, 1976). Three homologous opioid receptor genes have been identified that encode each of these receptor subtypes (Kieffer, 1995). Of these three, the δ-subtype seems to be the most effective for mediating analgesia while minimizing deleterious effects related to the μ-subtype such as addiction, respiratory depression, and constipation (Rapaka and Porreca, 1991; Quock et al., 1999). Opioid receptors are prototypical G_i/G_o-coupled receptors, as determined from the fact that opioid signals are efficiently blocked by pertussis toxin, a bacterial toxin that ADP-ribosylates and inactivates the α-subunits of G_i/G_o proteins (Law et al., 2000). The human δ-opioid receptor (hDOR) has been cloned (Knapp et al., 1994)

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ABBREVIATIONS: GPCR, G-protein-coupled receptor; hDOR, human δ-opioid receptor; CHO, Chinese hamster ovary; DPDPE, *c*-[D-Pen^2, D-Pen^5]enkephalin; PWR, plasmon-waveguide resonance; egg PC, egg phophatidylcholine; NTI, naltrindole; POPG, 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoglycerol; TIPP, H-Tyr-tetrahydroisoquinoline-3-carboxylic acid H-Tyr-Tic-Phe-Phe-OH; TMT-Tic, (2*S*,3*R*)β-methyl-2′, 6′-dimethyltyrosyl-tetrahydroisoquinoline-3-carboxylic acid; SNC80, SNC80, (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide; Tan 67, 2-methyl-4aα-(3-hydroxyphenyl)-1,2,3,4,4a,5,12,12aα-octahydroquinolino-[2,3,3-*g*]-isoquinoline; TM, transmembrane.

and characterized after being stably transfected in Chinese hamster ovary (CHO) cells (Okura et al., 2000).

High-resolution structural analysis of GPCRs has been hindered by their low natural abundance and the difficulty in producing, purifying, and crystallizing significant quantities of tissue-derived or recombinant protein. A recently developed method, plasmon-waveguide resonance (PWR) spectroscopy, is a highly sensitive technique (requiring as little as femtomole quantities of material) and has been very successfully used in our laboratories as a method for the characterization of anisotropic proteolipid membranes (Salamon et al., 1998, 2000, 2002; Alves et al., 2003; Devanathan et al., 2004). The technique allows real-time measurements of the binding of free molecules to immobilized ones (in the present case, integral membrane proteins inserted into a solid-supported lipid bilayer), without the application of specific labels. No labeling is required because the method is dependent on the intrinsic optical properties (refractive index, optical absorption coefficient) of material deposited onto the resonator surface. PWR has the unique capability of independently examining changes in the structure of the proteolipid film both parallel and perpendicular to the membrane plane in response to receptor-ligand interactions. It possesses several significant advantages compared with conventional surface plasmon resonance, including enhanced sensitivity and spectral resolution and the ability to distinguish between mass and conformational changes (Salamon et al., 1997). The latter is a consequence of the use of both *p*- and *s*-polarized excitation (electric vectors perpendicular and parallel to the resonator surface, respectively) to produce resonances, whereas surface plasmon resonance only responds to *p*-polarized excitation. This allows for measurements of refractive index anisotropy, which reflects changes in mass distribution and therefore changes in molecular orientation and conformation. Using this approach in the present study, we incorporated the hDOR into a solid-supported lipid bilayer and directly monitored in real time the conformational changes produced upon binding to the receptor of different classes of ligands [i.e., agonists (peptide and nonpeptide), partial agonists, antagonists, and inverse agonists]. The spectral changes and their kinetic properties were unique to a given ligand type, and the binding constants were found to correlate very well with values determined by radiolabeling binding assays. The results have provided new insights into signal transduction by this receptor and point the way toward high information content screening protocols that should prove useful in drug development.

Materials and Methods

Materials. The lipids used to prepare the lipid bilayer, egg PC and 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoglycerol (POPG), were purchased from Avanti Polar Lipids (Alabaster, AL). The peptides DPDPE (Mosberg et al., 1983), [*p*-Cl-Phe^4]-DPDPE (Toth et al., 1990), Deltorphin II (Misicka et al., 1991), and (2*S*,3*R*)TMT-Tic (Liao et al., 1997) were prepared by standard methods of solid-phase peptide synthesis (Hruby and Meyer, 1998). Naltrindole, naloxone, and morphine were purchased from Sigma Chemical (St. Louis, MO); TIPP was obtained from the National Institute of Drug Abuse as part of the drug distribution to National Institute on Drug Abuse grantees (to V.J.H.); SNC80 was purchased from Tocris Cookson Inc. (Ellisville, MO); Tan 67 was obtained from Toray Industries (Kumakura, Japan). The protected amino acids used in the synthesis of the ligand affinity resin were purchased from Bachem California (Torrance, CA).

Peptide Affinity Ligand for hDOR Purification. The ligand resin was prepared with the following sequence attached to a CM Sepharose resin: H-Tyr-*D*-Ala-Phe-Glu-Val-Val-Gly-β-Ala-Gly-β-Ala-Gly-resin, in which the first four amino acids attached to the solid support function as a spacer arm and the rest of the sequence corresponds to Deltorphin II, a potent and selective ligand for the hDOR. The Nα-Fmoc strategy of solid-phase peptide synthesis was used. The Gly residue was coupled to Sepharose resin (CM Sepharose; Pharmacia, Peapack, NJ) (0.09–0.13 mmol/ml) using 10 Eq Nα-Fmoc-Gly, 10 Eq hydroxybenzotriazlole, 10 Eq diisopropylcarbodiimide, and 4 Eq *N*-methylimidazole dissolved in a minimal amount of dimethylformamide. The resin was reacted with the previous mixture in a rocking platform for 1 h. A Kaiser test was performed to test for coupling reaction completion. The Nα-Fmoc group was cleaved by treating the resin with 25% piperidine in dimethylformamide for 30 min, and the absorbance was monitored at 302 nm to determine the amino substitution achieved. These steps were repeated for all the amino acids in the sequence. After coupling all of the amino acids to the resin, cleavage of the Nα-Fmoc group from the N terminus and cleavage of the side-chain protecting groups was achieved using 95% trifuoroacetic acid, 2.5% thioanisole, and 2.5% anisole for 1.5 h. To test the quality of the peptide, a small part of the peptide was cleaved from the resin using 0.5% NaOH in water during 30 min. The filtrate obtained was then submitted for mass spectral analysis which confirmed that the target peptide was synthesized in approximately 95% purity.

Construction of the hDOR Plasmid and Stable Transfection of CHO Cells. A fully functional receptor labeled at the C terminus with an myc epitope and His tag was prepared by inserting the DNA of the human δ-opioid receptor, which was modified by incapacitating the stop codon of the receptor, into the pcDNA3 vector containing the myc/His tag (Invitrogen, Carlsbad, CA). The entire vector was verified by DNA sequencing and stably transfected into a CHO cell line with the use of DEAE-dextran (Promega, Madison, WI). The transfected clones were selected using G418 as an antibiotic. Experiments characterizing the modified receptor have been carried out previously (Okura et al., 2000).

Cell Culture. CHO cells were cultured in Ham's F-12 medium (Invitrogen) supplemented with 10% of fetal bovine serum (Invitrogen), penicillin (100 units/ml) (Sigma), and streptomycin (100 μg/ml) (Sigma) in a humidified atmosphere containing 5% CO_2 at 37°C.

Preparation of Membrane Fraction from CHO Cells. Confluent monolayers of CHO cells, grown in 15-cm dishes, were washed with Ham's F-12 basic media and harvested using 5% trypsin in F-12. After centrifugation (2500 rpm for 20 min at 4°C), the pellet was resuspended in 25 mM Tris-HCl, pH 7.4, containing protease inhibitors (1 ml/l) that are designed to be used with metal chelating columns (Sigma). Cell lysates were homogenized by 10 strokes with a tissue grinder. The homogenates were centrifuged at 42,000*g* (Beckman Coulter, Fullerton, CA) for 30 min at 4°C to remove nuclei and debris. Supernatants were aspirated, and the membrane-enriched pellets were suspended in buffer (50 mM Tris-HCl, 0.15 M NaCl, 2 mM $CaCl_2$, 5 mM KCl, 5 mM $MgCl_2$, 4 mM EDTA, and 20% glycerol, pH 7.5) with the protease inhibitors added and stored at −80°C. Receptors in these crude membrane preparations were stable for at least 1 month under these conditions.

Solubilization and Purification of the hDOR. The crude membrane preparations containing the hDOR were resuspended in the solubilization buffers: 25 mM HEPES, 0.5M KCl, and protease inhibitors (1 ml/l) with either 30 mM of octylglucoside (Sigma) or 1% dodecylmaltoside (Calbiochem, San Diego, CA) in the absence or presence of 0.1 nM of the antagonist naltrindole (NTI) at a concentration of 10 ml/g membrane pellet. The membranes were then homogenized by 15 strokes and centrifuged at 42,000*g* (Beckman Coulter). This allowed the separation of a small amount of insoluble material from the supernatant. The supernatant was added to a

Nickel chelating resin (His-Select HC Nickel affinity gel; Sigma) (0.5 ml of resin per gram of lysed cells) in a 10-ml polypropylene column (Pierce, Rockford, IL), pre-equilibrated with the same buffer system as the hDOR preparation, and placed on a rocker platform for 1 h at 4°C. The resin was incubated first (20 min) with the detergent buffer containing 30 mM imidazole to remove nonspecifically bound proteins. The resin was washed once with three column volumes of detergent buffer followed by elution with detergent buffer containing 100 mM imidazole.

A second purification was performed using ligand affinity chromatography. The Deltorphin II resin was stored with buffer containing sodium azide and could be reused several times. Before using, the resin was washed with five column volumes of low-salt (0.1 M KCl) detergent buffer and then incubated with the His-tag–purified receptor for 2 h at 4°C. The affinity resin was then washed three times with one column of high-salt detergent buffer (0.5 M KCl), three times with one column of no-salt detergent buffer, and three times with one column of high-salt detergent buffer. The resin was then suspended in high-salt detergent buffer containing 0.1 nM NTI for 1 h, and the receptor was eluted.

Quantification of the hDOR. A bicinchoninic acid assay was performed to determine the protein concentration in the sample (Pierce). The purple reaction product was monitored at 560 nm using an enzyme-linked immunosorbent assay plate reader (μ Quant; Bio-Tek Instruments, Winooski, VT).

Radioreceptor Assay. After purification, the quality of the receptor protein was assessed by determining the specific activity (i.e., the number of functional receptor molecules, measured by ligand binding, per amount of receptor protein). Binding was performed by diluting the purified receptor to a final concentration of approximately 400 nM in low-salt buffer and adding [^{3}H]naltrindole (PerkinElmer Life and Analytical Sciences, Boston, MA) to a final concentration of approximately 0.1 nM. The binding reaction was incubated for 1 h at room temperature. Unbound ligand was then separated from the ligand-receptor complex by ultrafiltration (YM 30.000, Centricon; Instrument Co., Ann Arbor, MI) using low-salt buffer. A competition assay was then performed using DPDPE with concentrations ranging from 10^{-3} to 10^{-9}M. This ligand, dissolved in low-salt buffer, was incubated at room temperature for 1 h with the solution containing the [^{3}H]naltrindole receptor complex obtained previously. Samples were then placed in scintillation vials filled with scintillation liquid and measured in a counter (Beckman Coulter). Binding results plotted with the help of Prism (GraphPad Software Inc., San Diego, CA) demonstrated that the solubilized receptor was active, as determined by its ability to bind DPDPE with a K_d value of 15 to 20 nM (Mosberg et al., 1983).

Preparation of Lipid Membranes and Incorporation of the hDOR. In this study, we used self-assembled solid-supported lipid membranes (Salamon and Tollin, 1991; Salamon et al., 1996). The method of preparation uses the same principles that govern the spontaneous formation of a freely suspended lipid bilayer membrane (called a black lipid membrane) (Mueller et al., 1962). This involves spreading a small amount of lipid solution across a 2-mm orifice in a Teflon block that separates the thin dielectric film (SiO_2) 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, 1999a,b). In these experiments, the lipid films were formed on the silica surface from the following membrane-forming solution: 7 mg/ml egg PC and POPG (75:25 mol/mol) in squalene/butanol/methanol (0.05:9.5:0.5, v/v/v). The incorporation of the human δ-opioid receptor into this solid-supported lipid bilayer was accomplished by introducing the detergent-solubilized hDOR into the aqueous compartment under conditions that dilute the detergent below the critical micelle concentration. At this point, the membrane protein spontaneously incorporates into the lipid bilayer. The aqueous compartment was filled with approximately 1 ml of 10 mM Tris buffer, pH 7.3, containing 0.5 mM EDTA and 10 mM KCl.

Ligand Addition to the Proteolipid System. Between 20 and 100 μl of ligand dissolved in the above-mentioned buffer system was added to the cell sample, such that after dilution in the cell chamber, the target concentration was achieved. The first concentration point was chosen to be approximately 1 order of magnitude lower than the published K_d value for that ligand. Incremental amounts of ligand were then added in a cumulative fashion, and the PWR spectra were acquired for each point when equilibrium was reached (i.e., when no further changes in the PWR spectra occurred). K_d values were obtained from plotting the resonance minimum position for the PWR spectra as a function of ligand concentration and fitting to the hyperbolic function that describes the 1:1 binding of a ligand to a receptor using GraphPad Prism (GraphPad Software). The kinetics of the formation and decay of intermediate states for the agonist (peptide and nonpeptide) and partial agonist were analyzed using Origin (OriginLab Corp., Northampton, MA), and rate constants were obtained by fitting the data to a two-exponential equation.

PWR Spectroscopy. The method is based on the resonant excitation by polarized light from a CW He-Ne laser (λ = 632.8 or 543.5 nm) passing through a glass prism under total internal reflection conditions of collective electronic oscillations (plasmons) in a thin metal film (Ag) deposited on the external surface of the prism, which is overcoated with a dielectric layer (SiO_2). The resonant excitation of plasmons generates an evanescent electromagnetic field localized at the outer surface of the dielectric film, which can be used to probe the optical properties of molecules immobilized on this surface (Salamon et al., 1997, 1999; Salamon and Tollin, 1999b). Resonance is achieved by varying the incident angle (α) slightly above the critical angle for total internal reflection. Because the resonance coupling generates electromagnetic waves at the expense of incident light energy, the intensity of totally reflected light is diminished. Thus, the angular dependence of the reflectance corresponds to a PWR spectrum. The resonance can be excited with light polarized with the electric vector either parallel (p) or perpendicular (s) to the incident plane, thereby allowing for characterization of the molecular organization of anisotropic systems such as biomembranes containing integral proteins (Salamon et al., 1996, 1998, 2000, 2002). Under the experimental conditions used in this work, the optical parameters obtained with p-polarization refer to the perpendicular direction, and those obtained with s-polarization to the parallel direction relative to the bilayer membrane surface. PWR spectra can be described by three parameters: α, the spectral width, and the resonance depth. These depend on the refractive index (n), the extinction coefficient (k), and the thickness (t) of the plasmon-generating and emerging media, the latter including a thin film deposited on the silica surface (a proteolipid membrane in the present work) in contact with an aqueous solution. Thin-film electromagnetic theory based on Maxwell's equations provides an analytical relationship between the spectral and optical properties of these media. This allows for the evaluation of n, k, and t uniquely for the three media (the plasmon generating medium, the proteolipid membrane, and the aqueous buffer solution) by nonlinear least-squares fitting of the theoretical spectra to the experimental one. In the present study, we are concerned only with the shifts and amplitudes of the resonance spectra, without any analysis using spectral fitting. Furthermore, the molecules used here do not absorb significantly at the excitation wavelength, and thus, the spectra reflect mainly the refractive index and the thickness of the proteolipid layer (i.e., mass density and mass distribution). For nonspherical molecules oriented uniaxially on the resonator surface, n values will be different for s- and p-polarization. This allows charac-

terization of anisotropy changes caused by alterations in molecular orientation and structure of the molecules in the proteolipid film.

Resonance spectra in this study were obtained using a Beta PWR instrument from Proterion Corporation (Piscataway, NJ) that records the relative reflectance versus the absolute angle with a resolution of 1 mdeg.

Results

Formation of the Lipid Bilayer and Receptor Incorporation. From Fig. 1, A and B, one can see that the formation of a lipid bilayer on the resonator surface leads to increases in the resonance angle position (145 mdeg for *p*- and 95 mdeg for *s*-polarized resonances) as well as a decrease in the depth of the PWR spectra, both for *p*- and *s*-polarizations. The increase in the resonance angle can be ascribed in part to an increase in the refractive index that results from a gain in deposited mass, which is as expected. The decrease in the resonance depth is related to the formation of a thin film of lipid on the silica surface, which again is expected as a consequence of bilayer formation. The spectral changes are also anisotropic; the changes in *p*-polarization are quite different from those for *s*-polarization because of the highly anisotropic optical properties of oriented lipid bilayers.

Receptor molecules were incorporated into the lipid bilayer by adding two aliquots (50 μl each) of a concentrated solution of hDOR (~1 nM) solubilized in 30 mM octylglucoside-containing buffer to the aqueous compartment (total volume, ~1 ml; final receptor concentration in the cell sample compartment, approximately 0.1 nM) of the PWR cell sample, diluting the detergent to a final concentration lower than the critical micelle concentration (~25 mM for octylglucoside). The receptor that was not incorporated into the lipid bilayer was removed by flowing buffer through the cell-sample compartment. The amount of incorporated receptor was approximately the same in the different experiments, as monitored by the magnitude of the PWR spectral shifts observed for *p*- and *s*-polarized light. From Fig. 1, A and B, one can see that the incorporation of the receptor into the bilayer leads to anisotropic increases in the resonance angle (190 mdeg shift for the *p*- and 130 mdeg shift for the *s*-polarized resonance) and in the spectral depth that are the result of an increase in the mass and the thickness of the bilayer. Because the receptor protrudes from both sides of the lipid bilayer, one should expect the bilayer to become thicker upon receptor incorporation. These results are consistent with those reported previously upon the incorporation of the hDOR into a lipid bilayer (Salamon et al., 2000), in which the thickness of the proteolipid system was found by spectral fitting to increase from 5.3 (Salamon et al., 1997) to 6.8 nm (the latter value corresponds to the dimension of the incorporated protein molecule perpendicular to the membrane plane). A thickness of the proteolipid system of ~6.8 nm correlates well with the size determined for rhodopsin from X-ray crystallography (Palczewski et al., 2000). Again, spectral shifts with *p*-polarization were larger than with *s*-polarization (indicating refractive index changes in the *p*-direction higher than for the *s*-direction), which is a consequence of the anisotropic structure (i.e., cylindrical shape) of the receptor molecules. This is also evidence for the incorporation of the receptor into the bilayer with the expected orientation (i.e., the long axis oriented perpendicularly to the lipid bilayer), rather than just adsorbed to the surface of the bilayer, clearly reflecting a corresponding increase of the average long-range molecular order in the membrane resulting from receptor-lipid interactions. From this and previous results involving interactions of the hDOR with ligands (Salamon et al., 2000) and with G-proteins (Alves et al., 2003), we presume that the receptor is incorporated bidirectionally into the lipid bilayer, with either the ligand binding site or the G-protein binding site facing the aqueous compartment of the PWR cell sample.

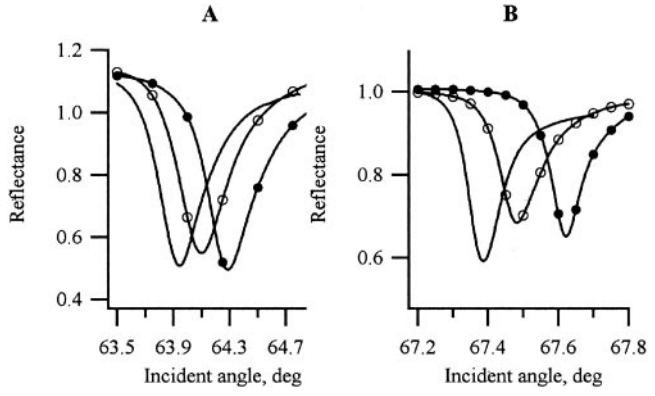

Fig. 1. PWR spectra obtained for lipid bilayer formation and receptor incorporation using *p*-polarized (A) and *s*-polarized (B) light excitation. Unlabeled solid lines represent the buffer spectra (10 mM Tris buffer, pH 7.3, 0.5 mM EDTA, and 10 mM KCl) before bilayer formation; ○, PWR spectra obtained after the formation of a lipid bilayer composed of 75:25 mol% egg PC/POPG; ●, PWR spectra obtained after the addition of an octylglucoside-containing buffer solution of hDOR; final concentration in the cell sample compartment ~0.4 nM.

Peptide Agonist Binding to the hDOR. When aliquots of the selective hDOR agonist DPDPE were added to the cell sample after the incorporation of hDOR into the lipid bilayer, significant changes in the position (−30 mdeg for *p*- and 10 mdeg for *s*-polarization), width, and depth of the PWR resonance curves occurred. These are shown in Fig. 2, A and B, and correspond to the addition of a saturating amount of the ligand. Table 1 shows that the PWR spectral shifts, obtained from three independent experiments, are quite reproducible. Control experiments were done in which similar concentrations of DPDPE were added to the lipid bilayer without having receptor incorporated, and no measurable effects were detected in the PWR spectra (data not shown). This demonstrates the absence of nonspecific ligand binding to the bilayer in these experiments. It should also be noted that the spectral changes saturated within concentration ranges that are consistent with literature data for the binding characteristics of the ligand (see below). The spectral changes obtained after the addition of ligand must then reflect receptor-ligand interactions.

In Fig. 2, A and B, anisotropic changes are seen in the resonance position, width, and spectral depth. Most dramatically, DPDPE shifted the resonance to smaller angles in the case of *p*-polarized light and to larger angles in the case of *s*-polarization. If one plots the resonance position minimum obtained for each incremental addition of ligand, one obtains a simple hyperbolic curve, as expected for single-site saturable binding (Fig. 2C). By fitting the data using a hyperbolic function, one can obtain dissociation constants for the receptor/ligand interaction that are very similar to values obtained using classic pharmacology procedures (Table 2). As expected, the K_d values obtained for *p*- and *s*-polarized excitation are the same within experimental error.

Time-resolved measurements (done with *s*-polarized light) were also made of the PWR spectral changes after ligand addition (Fig. 2D). There are two features of the kinetics that should be pointed out: the overall spectral changes occurred with time constants on the order of minutes, and they were biphasic, involving negative shifts followed by positive shifts. This is similar to what was observed previously using PWR (Salamon et al., 2000) and indicates that agonist binding is a mechanistically complex process involving anisotropic structural changes in the receptor-lipid system, with at least one distinguishable intermediate state involved. It was suggested that the slowness of the conformational change was related to the fact that the lipid bilayer surrounding the receptor has to rearrange in response to the receptor conformational change. The apparent first-order rate constants obtained by fitting the data for the formation (k_1) and for the disappearance of the intermediate state (k_{-1}) were comparable at 0.28 and 0.25 min^{-1}, respectively (Table 3).

Results quite similar to these in terms of the PWR spectral shifts (Table 1), especially relative to the anisotropic properties of the changes, were obtained with two other peptide agonists, [*p*-Cl-Phe^4]-DPDPE and Deltorphin II; K_d values for ligand binding were obtained that are comparable with those obtained by classic radiolabeling methods and are given in Table 2. The apparent first-order rate constants obtained for the formation and disappearance of the intermediate state are given in Table 3, and they are within experimental error the same as for DPDPE.

Nonpeptide Agonist Binding to the hDOR. Using the same strategy as above, a nonpeptide agonist (Tan 67) was added to the proteolipid system after hDOR incorporation. As can be seen in Fig. 3, A and B, the addition of this ligand to the proteolipid system shifted both resonances to larger incident angle values (13 mdeg for *p*- and 9 mdeg for *s*-polarization). These results indicate that the interactions of the hDOR with peptide and nonpeptide agonists generate different structural states of the proteolipid membrane. As with DPDPE, by plotting the resonance position shift versus the ligand concentration (Fig. 3C and Table 1), K_d values were obtained (Table 2) that are comparable with those determined by classic methods. Time-resolved experiments for the nonpeptide agonist also show some differences relative to the peptide agonist; although biphasic kinetics were again observed, the apparent first-order rate constants for the formation and the disappearance of the intermediate state were quite different from each other (k_1 being approximately twice the value of k_{-1}), contrary to what was observed in the peptide agonist case (Table 3). Results similar to these, both in terms of the direction of the PWR spectral changes (Table 1) and the kinetic data (Table 3), were also obtained with SNC80, another nonpeptide agonist.

Antagonist Binding to the hDOR. The PWR spectral changes observed upon a nonpeptide antagonist (NTI) addition after hDOR incorporation, shown in Fig. 4, A and B, were again different from those obtained for the agonist case (both peptide and nonpeptide). Thus, NTI addition causes shifts in the PWR spectra to smaller angles for both *p*- and *s*-polarization (−50 mdeg for *p*- and −20 mdeg for *s*-polarization) (Table 1). This clearly demonstrates that a different receptor conformation was generated. These shifts were quite large and were larger for *p*- than for *s*-polarization, denoting an anisotropic structural change. Also noteworthy are the increases in the spectral depth and decreases in spectral width, especially in the case of *s*-polarization spectra. In contrast to the agonist case, antagonist addition resulted in spectral changes that were faster than the resolution time of the instrument, occurring in fewer than 5 s. This is again similar to the results obtained earlier (Salamon et al., 2000). The kinetic patterns obtained for agonist and antagonist show striking parallels to studies performed with

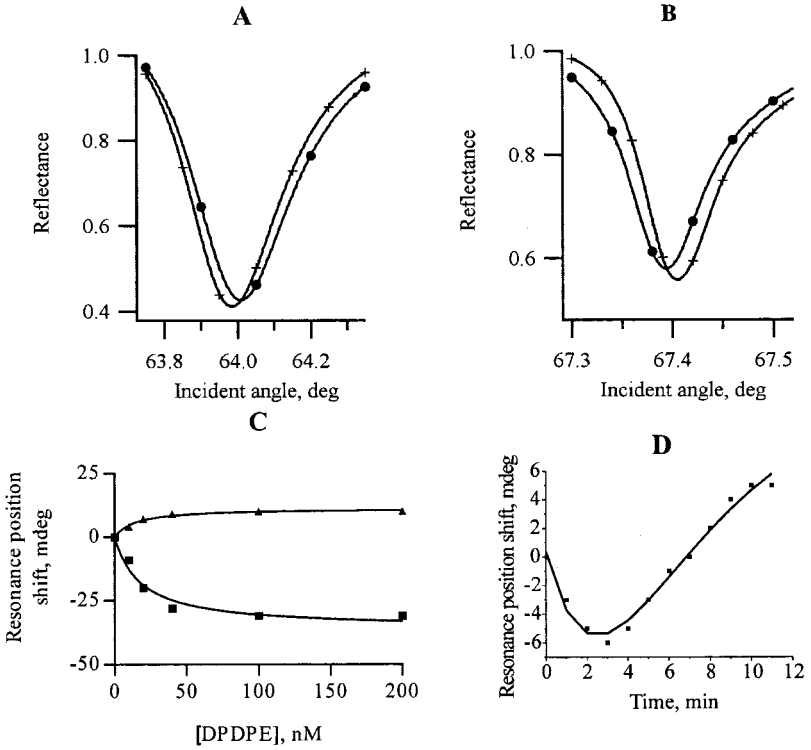

Fig. 2. PWR spectral changes obtained upon the addition of peptide agonist (DPDPE) to the proteolipid system after hDOR incorporation using *p*- (A) and *s*-polarization (B). ●, spectra for bilayer after receptor incorporation; +, spectra obtained upon addition of 200 nM (saturating concentration) of DPDPE to the proteolipid system. C, plots of resonance position shift obtained using *p*- (■) and *s*-polarization (▲) versus concentration of ligand, from which K_d values were obtained. D, kinetic data obtained for the formation of the intermediate state observed using *s*-polarized light. Symbols represent the data points, and the solid line is the best fit obtained using the following equation: $y = a0 + a1 \exp(-k_1 x) + a2 (1 - \exp(-k_{-1} x))$, where k_1 represents the rate constant for the formation and k_{-1} represents the rate constant for the disappearance of the intermediate.

the β_2 adrenergic receptor, in which fluorescence spectroscopy (Gether et al., 1997, Swaminath et al., 2004) and PWR (Devanathan et al., 2004) were used to delineate structural changes associated with receptor-ligand interactions. Plots of the spectral shifts versus NTI concentration are shown in Fig. 4c; the K_d values obtained (Table 2) are consistent with literature values. Similar results in terms of the direction of the PWR spectral shifts were obtained with another nonpeptide antagonist, Naloxone, and with a peptide antagonist (TIPP) (Table 1) with affinity values comparable with those reported in literature (Table 2).

Inverse Agonist Binding to the hDOR. When the inverse agonist TMT-Tic was added to an hDOR-containing bilayer, the resulting spectral changes (Fig. 5, A and B) resulted in increases to higher angles both for *p*- and *s*-polarizations (5 mdeg for *p*- and 14 mdeg for *s*-polarized light) (Table 1). This is similar to what was seen in the case of the nonpeptide agonist (compare with Fig. 3, A, B, and C). It should be noted, however, that in the case of the nonpeptide agonist the magnitudes of *p*- and *s*-shifts were quite similar (with *p*- slightly larger than *s*-polarization), whereas in the present case the shifts obtained with *s*-polarization were much larger than those obtained with *p*-polarization (Table 1). This demonstrates that the anisotropy changes were quite different, again reflecting differences in the structural states generated by ligand binding. It is also important to emphasize that, even though the spectral shifts obtained with *p*-polarized light were small, they are significant because they were larger than the instrumental resolution (1 mdeg), and they also saturate as a function of ligand concentration (Fig. 5c). Note also the small but significant amplitude changes and their reproducibility (Table 1). Again, the K_d values (Table 2) are similar to those obtained by classic methods. The kinetics of the formation of the inverse agonist receptor conformational state were comparable with those of the antagonist case; they occurred within the instrument response time.

Partial Agonist Binding to the hDOR. The addition of the partial agonist, morphine, to the hDOR (Fig. 6, A and B) produced shifts in the PWR spectral lines to smaller angles with *p*-polarized light (−18 mdeg for *p*-polarized light) but no net change in spectral position with *s*-polarization (Table 1). In this case, kinetic experiments showed that there was initially a shift in the *s*-polarized resonance position to smaller angles followed by a shift to higher angles, with the final position being indistinguishable from the initial one in terms of resonance angle position. Similar results were obtained with the related compound ethorphine (Table 1). The apparent first-order rate constants k_1 and k_{-1} were similar to each other and to the values for the full agonists for both partial agonists tested (morphine and ethorphine) (Table 3). Furthermore, there was a change in the spectral amplitude for the *s*-polarized resonance despite the absence of a net shift (Fig. 6B), indicating an overall conformational change in the *s*-plane. It is quite interesting that this behavior is significantly different from those observed with the peptide and nonpeptide agonists and the antagonists. Thus, it would seem that the partial agonist induced a conformation in the receptor that had properties intermediate to those of a peptide agonist and an antagonist; the behavior in terms of resonance position resembled the peptide agonist case, whereas the spectral amplitude changes were more comparable with those obtained in the antagonist case. K_d values were obtained (Table 2) that are quite similar to those determined by radiolabeling methods.

TABLE 1

Magnitude of the PWR spectral shifts obtained upon binding of ligand to the hDOR incorporated into a lipid bilayer

The PWR spectral shifts presented are the average obtained from three independent experiments.

Ligands	PWR Spectral Shifts upon Ligand Binding	
	p-Polarization	*s*-Polarization
	mdeg	
DPDPE	−25 ± 5	10 ± 2
pCl-DPDPE	−20 ± 2	7 ± 1
Deltorphin II	−14 ± 2	9 ± 2
SNC80	13 ± 2	6 ± 2
Tan 67	14 ± 2	9 ± 2
TIPP	−26 ± 4	−13 ± 3
NTI	−50 ± 8	−20 ± 3
Naloxone	−24 ± 5	−12 ± 3
TMT-L-Tic	4 ± 1	14 ± 2
Morphine	−18 ± 2	
Ethorphine	−15 ± 2	

Empty cells indicate that no overall PWR spectral shifts were observed.

TABLE 2

Affinity constants for binding of the different ligands to the hDOR obtained in these studies using PWR and from previous literature

K_d values were obtained from plotting the resonance minimum position (Y) for the PWR spectra as a function of ligand concentration (X) and fitting to the following hyperbolic function that describes the binding of a ligand to a receptor: $Y = (B_{max} X)/(K_d + X)$. B_{max} represents the maximum concentration bound, and K_d is the concentration of ligand required to reach half-maximal binding.

Ligands	K_d Obtained from PWR Experiments		Ligand Affinities Obtained from Literature	
	p-Polarization	*s*-Polarization		
	nM		*nM*	
DPDPE	14 ± 3	18 ± 5	16	(Mosberg et al., 1983)
pCl-DPDPE	2.9 ± 0.7	3.3 ± 0.8	1.6	(Toth et al., 1990)
Deltorphin II	0.88 ± 0.05	1.2 ± 0.3	0.7	(Misicka et al., 1991)
SNC80	52 ± 8	57 ± 12	56	(Hosohata et al., 2001)
Tan 67	3.2 ± 1.2	3.7 ± 1.5	6	(Nagase et al., 1998)
TIPP	1.1 ± 0.1	1.2 ± 0.1	1.22	(Schiller et al., 1999)
NTI	0.025 ± 0.001	0.023 ± 0.004	0.028	(Knapp et al., 1994)
Naloxone	8 ± 3	8 ± 1	10	(Lewanowitsch et al., 2003)
TMT-L-Tic	2.5 ± 0.3	3.2 ± 0.2	9	(Liao et al., 1997)
Morphine	520 ± 30		1101	(Gharagozlou et al., 2002)
Ethorphine	0.3 ± 0.1		0.2	(Gharagozlou et al., 2002)

Empty cells indicate that no net spectral shifts were obtained for this ligand using *s*-polarized light.

Discussion

As demonstrated by the above results, different classes of ligands produced distinctly different spectral changes (shift directions, spectral amplitudes, and kinetics) upon binding to the hDOR. Such different spectral changes cannot be explained simply by differences in either the adsorbed mass of the ligand or its rate of diffusion to the receptor. Thus, these ligands all have similar molecular masses, and the refractive index changes induced by ligand binding are too large to be accounted for by the added ligand mass. Furthermore, in those cases in which more than one example was used, the pattern of spectral changes and their kinetic properties were similar among ligands belonging to the same class, indicating that each class of ligand produced a distinguishable conformational state and induced structural changes of the receptor in a different and very specific way. It should be noted that the kinetics for the formation and decay of an intermediate state in the case of agonists and partial agonists were quite similar (Table 1), even though the calculated affinities of those ligands to the receptor were different (Table 2). The affinity differences must then be a consequence of their overall association and dissociation rates being different. Additional structural details of both the final and intermediate conformational states can be obtained by performing either graphical (Salamon and Tollin, 2004) or spectral fitting analyses (Salamon et al., 2000, 2002) and will be presented in a separate publication.

Conformational changes by GPCRs have been investigated in the last decade by different laboratories using different spectroscopic and biochemical techniques. Rhodopsin has been used as a model for studying the structure and mechanisms of activation of GPCRs. Several spectroscopic techniques have been applied to rhodopsin that have consistently provided evidence for a significant conformational rearrangement accompanying the transition of rhodopsin to the acti-

TABLE 3

Apparent first-order rate constants obtained for the formation (k_1) and decay (k_{-1}) of an intermediate state that occurs between the unoccupied and ligand-bound state of the hDOR

Rate constants were obtained using Origin software by fitting the data to the following double-exponential equation: $y = a0 + a1 \exp(-k_1 x) + a2 (1 - \exp(-k_{-1} x))$.

Ligands	Apparent First-Order Rate Constants	
	k_1	k_{-1}
	min^{-1}	
DPDPE	0.28 ± 0.05	0.25 ± 0.04
pCl-DPDPE	0.27 ± 0.06	0.26 ± 0.04
Deltorphin II	0.28 ± 0.05	0.27 ± 0.05
SNC80	0.30 ± 0.09	0.17 ± 0.08
Tan 67	0.28 ± 0.09	0.15 ± 0.06
Morphine	0.21 ± 0.08	0.20 ± 0.05
Ethorphine	0.19 ± 0.08	0.20 ± 0.07

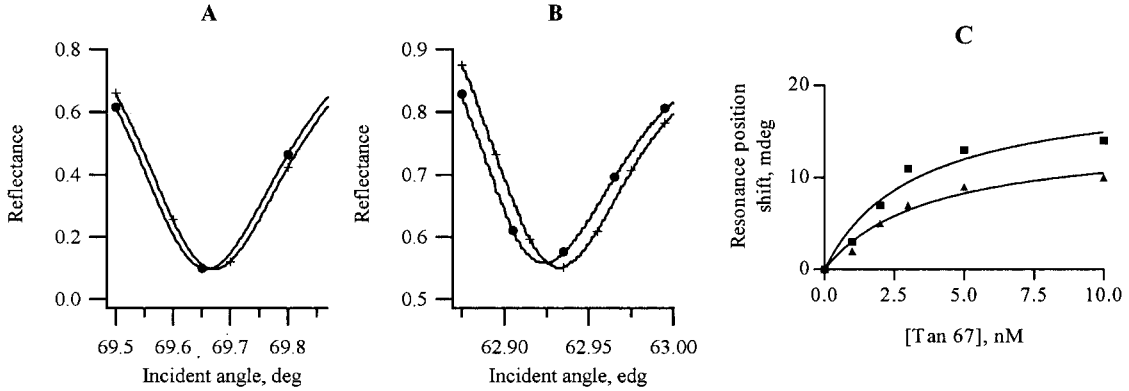

Fig. 3. PWR spectral changes obtained upon nonpeptide agonist (Tan 67) addition to the proteolipid system after hDOR incorporation using *p*- (A) and *s*-polarization (B). ●, spectra for bilayer after receptor incorporation; +, spectra obtained upon addition of 10 nM (saturating concentration) of Tan 67 to the proteolipid system. C, plots of resonance position shift obtained using *p*- (■) and *s*-polarization (▲) versus concentration of ligand, from which K_d values were obtained.

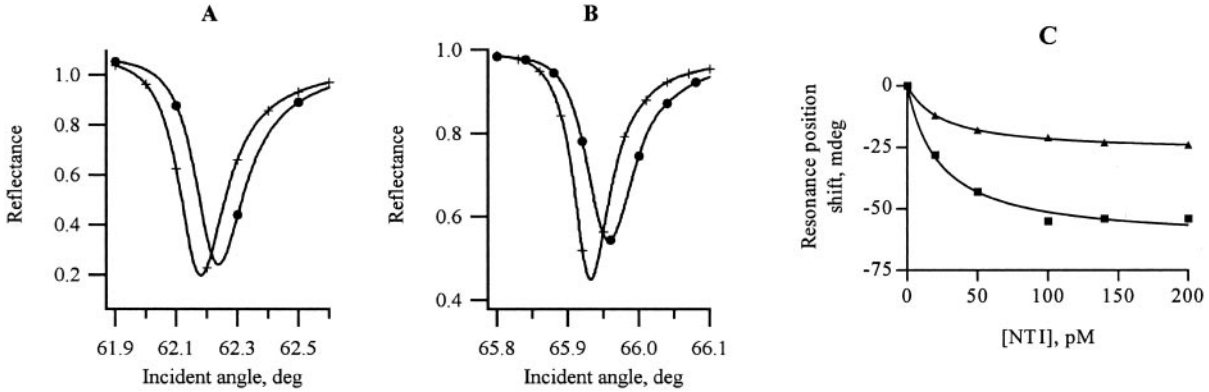

Fig. 4. PWR spectral changes obtained upon nonpeptide antagonist (NTI) addition to the proteolipid system after hDOR incorporation using *p*- (A) and *s*-polarization (B). ●, spectra for bilayer after receptor incorporation; +, spectra obtained upon addition of 200 pM (saturating concentration) of NTI to the proteolipid system. C, plots of resonance position shift obtained using *p*- (■) and *s*-polarization (▲) versus concentration of ligand, from which K_d values were obtained.

vated metharhodopsin II state (Farrens et al., 1996; Altenbach et al., 1999a,b; Dunham and Farrens, 1999). These studies point to a relatively small movement of TM3 and significant rigid-body movement of TM6 in a counter-clockwise direction (as viewed from the extracellular side) and a movement of the cytoplasmic end of TM6 away from TM3. Light-induced conformational changes also have been observed in the cytoplasmic domain spanning TM1 and TM2 (Altenbach et al., 1999a) and in the cytoplasmic end of TM7 and helix 8 (Altenbach et al., 1999b). Structural studies using fluorescent techniques to obtain insight into the β_2 adrenergic receptor agonist-induced conformational changes also have found that motions of TM3 and TM6 are involved in receptor conformational changes (Gether et al., 1997). Random mutagenesis studies of the hDOR (Decaillot et al., 2003) have suggested an activation path originating from the third extracellular loop and propagating through tightly packed TM3, TM6, and TM7 down to a TM6 to TM7 cytoplasmic switch.

The idea that GPCRs adopt multiple conformations has been proposed previously by several researchers in this area (Kobilka and Gether, 1998; Kenakin, 2002; Kobilka, 2002), but very few experimental methods have allowed detailed insights into the changes in molecular orientation and structure. It should be kept in mind, however, that PWR spectra reflect changes occurring in the entire proteolipid membrane; contributions from both lipid and protein molecules occur. Although it is not clear from the present studies what the precise structural basis for these differences is, the relative magnitudes of the *s*- and *p*-polarized shifts provide insights into the symmetry of the conformation changes. Despite the lack of details concerning structure, these results clearly have implications for drug discovery protocols. Thus, the different patterns produced upon ligand binding provide a direct and nonradioactive method to distinguish between pharmacologically different classes of ligands, thereby providing a complementary new way of doing drug screening. It also should be noted that, in a single experiment, information about both the binding affinity and the type of ligand can be obtained, whereas using classic pharmacological methods, two independent experiments are needed to obtain such information.

It is important to point out that previous PWR studies done in our laboratories have also demonstrated that agonists, antagonists, and inverse agonists produce different conformational states in the hDOR (Salamon et al., 2000, 2002), and the present work has extended this to include a larger group of ligands. However, some of the spectral changes obtained before were qualitatively different from the ones presented in this study (e.g., DPDPE and NTI). We attribute these differences to the fact that the starting points in terms of the receptor conformational state were different as a con-

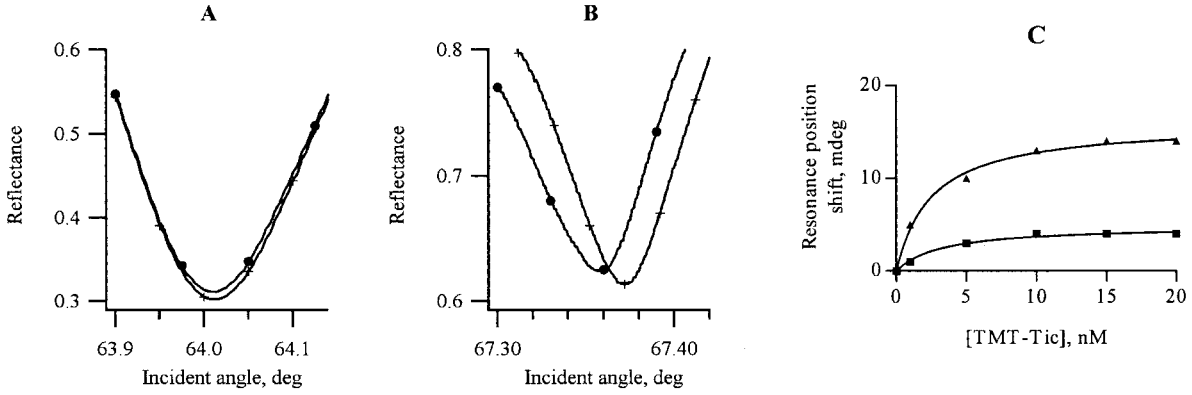

Fig. 5. PWR spectral changes obtained upon inverse agonist (TMT-Tic) addition to the proteolipid system after hDOR incorporation using *p*- (A) and *s*-polarization (B). ●, spectra for bilayer after receptor incorporation; +, spectra obtained upon addition of 20 nM (saturating concentration) of TMT-Tic to the proteolipid system. C, plots of resonance position shift obtained using *p*- (■) and *s*-polarization (▲) versus concentration of ligand, from which K_d values were obtained.

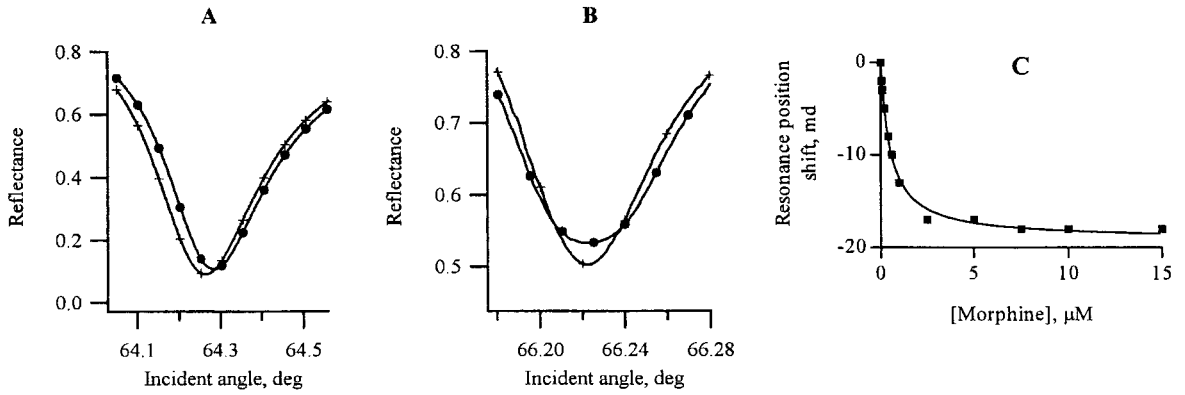

Fig. 6. PWR spectral changes obtained upon partial agonist (morphine) addition to the proteolipid system after hDOR incorporation using *p*- (A) and *s*-polarization (B). ●, spectra for bilayer after receptor incorporation; +, spectral change obtained upon addition of 15 μM (saturating concentration) of morphine to the proteolipid system. C, plots of resonance position shift obtained using *p*- (■) and *s*-polarization (▲) versus concentration of ligand from which K_d values were obtained.

sequence of differences in the purification protocols. Thus, in the previous studies, the hDOR was purified by a single step involving a metal chelating column. Because we have found this receptor preparation to be rather unstable, we modified our purification protocol to improve both the purity and stability of the sample. The present solubilization/purification procedure includes three steps that we believe affect the initial receptor structural state: 1) the CHO cells expressing the hDOR were grown in the presence of NTI, and this ligand was present during the solubilization procedure; 2) we used a ligand affinity column (Deltorphin II, see *Materials and Methods* for details) to further purify the receptor; this would be expected to induce the receptor into an agonist-type conformation; and 3) the receptor was eluted from the ligand affinity column using an excess of the antagonist NTI. Thus, in the present experiments, the starting point in terms of receptor occupancy and conformation is different from that in our previous experiments; the receptor was exposed to both agonist and antagonist before the PWR experiment. As noted in our previous work (Salamon et al., 2000), in some cases the final receptor state achieved was very much dependent on the ligand the receptor had originally seen. This is an important point that must be taken into account in studies involving GPCRs. Another important point that is emerging from our work is that the patterns of ligand-induced conformational transitions observed here for the hDOR are different for at least one other GPCR, the β_2 adrenergic receptor (Devanathan et al., 2004), although again, different classes of ligands produce unique structural states with unique kinetic properties.

In summary, we believe this methodology will be quite useful in unraveling many aspects of membrane signal transduction, as well as for drug development protocols. Future extensions of these studies to include other aspects of receptor behavior, such as G-protein interactions (Alves et al., 2003) and receptor down-regulation through β-arrestin interactions, are now in progress.

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