Direct Observation of G-protein Binding to the Human δ-Opioid Receptor Using Plasmon-Waveguide Resonance Spectroscopy*"§

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Using a recently developed method (Salamon, Z., Macleod, H. A., and Tollin, G. (1997) Biophys. J. 73, 2791–2797), plasmon-waveguide resonance spectroscopy, we have been able, for the first time, to directly measure the binding between the human brain δ-opioid receptor (hDOR) and its G-protein effectors in real-time. We have found that the affinity of the G-proteins toward the receptor is highly dependent on the nature of the ligand pre-bound to the receptor. The highest affinity was observed when the receptor was bound to an agonist (−10 nM); the lowest when receptor was bound to an antagonist (−500 nM); and no binding at all was observed when the receptor was bound to an inverse agonist. We also have found direct evidence for the existence of an additional G-protein binding conformational state that corresponds to the unliganded receptor, which has a G-protein binding affinity of −60 nM. Furthermore, GTP binding to the receptor-G-protein complex was only observed when the agonist was pre-bound. Similar studies were carried out using the individual G-protein subtypes for both the agonist and the unliganded receptor. Significant selectivity toward the different G-protein subtypes was observed. Thus, the unliganded receptor had highest affinity toward the G_12 (Kd = 20 nM) and lowest affinity toward the G_12 (Kd = 200 nM) subtypes, whereas the agonist-bound state had highest affinity for the G_12, and G_12 subtypes (Kd = 9 nM and 7 nM, respectively). GTP binding was also highly selective, both with respect to ligand and G-protein subtype. We believe that this methodology provides a powerful new way of investigating transmembrane signaling.

Opioid receptors belong to the superfamily of GPCRs.1 Their predicted topology is that of a polytopic integral membrane protein with seven membrane-spanning helical segments, an extracellular N terminus and an intracellular C terminus. These receptors and their endogenous ligands, opioid peptides such as endorphins and enkephalins, form a neuromodulatory system that is involved in stress-induced analgesia, that affects locomotive activity and regulates neuroendocrine physiology and autonomic functions such as respiration, blood pressure, and gastrointestinal motility. The opioid system has also been shown to play a role in learning and memory and possibly in the modulation of the immune system (1) and is an important factor in pain modulation and drug abuse. Studies to determine the G-protein subtypes that mediate the intracellular signaling of the opioid receptor systems have shown that functional coupling occurs to the G_i-G_s family of G-proteins (2–4). The human brain δ-opioid receptor has been cloned (5), stably transfected in Chinese hamster ovary (CHO) cells, and characterized (6).

Due to their integral membrane nature and their low cellular concentrations, there is little information on the structures and functional mechanisms of the opioid receptors. Furthermore, classic pharmacological methods only give indirect information about the interaction of GPCRs with G-proteins, because they are based on downstream responses. The GTPγS assay reflects a combination of both the affinity of the G-protein to the ligand-bound receptor (7) and the ability of the agonist-bound state to initiate GDP dissociation from the G-protein (8). Thus, such measurements do not directly probe interactions between the various signal transduction partners and suffer the disadvantages of being very time consuming and dependent on the use of radiolabeled material. Another limitation of such experiments comes from the fact that cells usually express all the different G-protein subtypes making it very hard to understand the contribution of each individual subtype.

PWR spectroscopy is a newly developed innovative experimental methodology that enables in-depth characterization of protein-protein, protein-lipid, and protein-ligand interactions occurring either within or at the surface of anisotropic thin films under native conditions and without the use of labels (9–14). By measuring plasmon resonances excited by light-polarized both parallel (s) and perpendicular (p) to the deposited film, this technology allows direct measurements of the anisotropic optical properties of biomembranes and real-time characterization of changes in the mass density and molecular orientation of molecules contained therein and thus can be used to monitor the thermodynamics and kinetics of binding processes and the accompanying structural changes. In the present study we have incorporated purified detergent-solubilized hDOR molecules into a lipid bilayer deposited onto the silica surface of a PWR resonator (13, 15) and have directly observed the interaction of a G-protein mixture with either the unliganded receptor or with receptor pre-bound with either the...
agonist DPDPE (c-[β-Pen², d-Pen⁶] enkephalin), the antagonist naltrindole (NTI), or the inverse agonist TMT-L-Tic. We have found that the affinity of G-proteins to the hDOR and the ability to bind GTP are very much dependent on the identity of the ligand that is pre-bound to the receptor. Similar studies were also done using the individual G-protein subtypes, and a high level of selectivity was observed that is highly modulated by the liganded state of the receptor. The results yield new insights into GPCR function and demonstrate that PWR provides a new, simple, and direct approach to investigate transmembrane signaling.

EXPERIMENTAL PROCEDURES

Peptide Affinity Ligand Synthesis and Use in hDOR Purification—
The ligand resin was prepared with the following sequence attached:

H-Tyr-D-Ala-Phe-Glu-Val-Ala-Gly-β-Ala-Gly-β-Ala-Gly-resin, where 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 DOR. The N⁵-Fmoc strategy of solid-phase peptide synthesis was used. The first Gly residue was coupled to Sepharose resin (CM-Sepharose, Amersham Biosciences) (0.09–0.13 mmol/ml) using 10 eq of N⁵-Fmoc-Gly, 10 eq of N⁵-hydroxybenzotriazole, 10 eq of N⁵-diisopropylcarbodiimide, and 4 eq of N⁵-methylimidazole dissolved in a minimal amount of dimethyl formamide. The resin was reacted with the previous mixture in a rocking platform for 1 h. The N⁵-Fmoc group was cleaved by treating the resin with 25% piperidine in dimethyl formamide during 30 min, and the absorbance at 302 nm was measured to determine the level of amino substitution achieved. These steps were repeated for all the amino acids in the sequence. After coupling all the amino acids, cleavage of the N⁵-Fmoc group from the N terminus, and cleavage of the side-chain protecting groups was achieved using 95% trifluoroacetic 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 resin using 0.5% NaOH in water during 30 min. The filtrate obtained was then submitted for mass spectral analysis that confirmed that the target peptide was synthesized in about 95% purity. The Deltorphin II resin was stored with buffer containing sodium azide and reused several times. The resin was washed with 5 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 1 column of high salt detergent buffer (0.5 M KCl), three times with 1 column of no salt detergent buffer and three times with 1 column of high salt detergent buffer. The resin was then suspended in high salt detergent buffer containing 0.1 M nastrindole for 1 h, and the receptor was eluted.

Receptor Purification and Characterization—A fully functional receptor, labeled at the C terminus with a myc epitope and His tag, was expressed in a Chinese hamster ovary cell line (CHO-K1), and the modified receptor was characterized (6). The receptor was purified using a modified version of a previously published method (13). Changes include use of 1% dodecyl maltoside instead of octylgycloside, the use of an antagonist (0.1 nM naltrindole, Sigma) during the solubilization and purification procedure and the use of ligand affinity chromatography as a second purification method. Details of the synthesis of this ligand affinity resin and its use, as well as the characterization of the solubilized receptor, can be found in the online Supplementary Material (Methods). For the present studies, the hDOR was preincubated with saturating amounts (at least one order of magnitude higher than the receptor concentration) of the antagonist BIM 23148 (Peptide Company, CA), a peptide agonist (TMT-L-Tic, Sigma) or an inverse agonist (TMT-L-Tic; synthesized in Dr. Hruby’s laboratory following published procedures (16)). The receptor was incubated with the respective ligand for 1–2 h at 4°C.

A BCA (bicinchoninic acid) assay was performed to determine the protein concentration in the sample (Pierce). The purple reaction product was measured at 560 nm using an equation relating to the absorbance of the assay plate reader (μQuant, Bio-Tek Instruments, Inc). Following purification, the quality of the receptor protein was assessed with determining the specific activity, i.e. the moles of functional receptor molecules (measured by ligand binding) per mole of receptor protein. Binding was performed by diluting the purified receptor to a final concentration of about 400 nM in low salt buffer. A competition assay was then performed using DPDPE with concentrations ranging from 10⁻⁸ to 10⁻² M. This ligand, solubilized in low salt buffer, was incubated with the solution containing the [³H]naltrindole-receptor complex prepared as previously reported at room temperature for 1 h. Samples were then placed in scintillation vials filled with scintillation liquid and measured in a counter (Beckman). Binding results were plotted using GraphPad Prism (San Diego, CA).

Lipid Bilayer Formation, hDOR Incorporation, and G-protein Addition—In this study we used self-assembled solid-supported lipid membranes (17, 18). 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) (19), as previously reported (13, 15). The lipid films were formed on the silica surface of the PWR resonator from the following membrane-forming solutions: 7 mg/ml egg PC and 1-palmitoyl-2-oleyl-sn-glycero-3-phosphoglycerol (Avanti Polar Lipids) (75:25 mol/mol) in squalene/butanol/methanol (0.05:0.95:0.5, v/v). The incorporation of the human δ-opioid receptor into this lipid bilayer was accomplished by introducing the detergent-solubilized hDOR into the aqueous compartment under conditions that dilute the detergent to below the critical micelle concentration, which allows the membrane protein to spontaneously incorporate into the lipid bilayer. In these experiments we were interested in studying the ternary complex, i.e. ligand, receptor, and G-protein. However, we do not have simultaneous access to both sides of the receptor, and we think that neither the G-protein nor most of the ligands are able to cross the bilayer. Since binding to both the G-proteins and ligands can occur after receptor incorporation, it appears that the receptor inserts bi-directionally into the lipid bilayer. Thus, we have studied the ternary complex by prebinding the receptor with the ligand before incorporation into the lipid bilayer. In this way, some of the ligand-bound receptors will have their G-protein binding sites accessible to the external aqueous medium. Small aliquots of G-proteins consisting of a purified mixture of the predominant forms of pertussis toxin-sensitive G-proteins, associated with the DOR, from bovine brain (Calbiochem) containing Ga (−4–5 μM), Gα₁ (−1–2 μM), Gα₁ (−1–2 μM), Gα₁ (−1 μM), and the βγ subunit complex (βγ5 μM) were incrementally added to the equilibrated proteolipid system. The purified individual G-protein subtypes were also obtained from Calbiochem. After saturation was reached, GTPγS (Sigma) was added and the PWR spectral changes were monitored.

Plasmon-Waveguide Resonance Spectroscopy—The method is based upon the resonant excitation by polarized light from a CW He-Ne laser (λ = 632.8 nm or λ = 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 (SiO2). 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 (9, 20, 21). Resonance is achieved either by varying the incident light wavelength ($\lambda$) at a fixed angle ($\phi$), or by varying $\phi$ at a fixed $\lambda$ (the latter protocol was used in our experiments). Because the resonance coupling generates electromagnetic waves at the expense of incident light energy, the intensity of totally reflected light is diminished at a specific angle. 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 (11, 14, 21). The experimental arrangement for PWR measurements is shown in Fig. 1. Under the experimental conditions employed in this work the optical parameters obtained with the $p$-polarization refer to the perpendicular direction, and those obtained with $s$-polarization refer to the parallel direction, relative to the bilayer membrane surface. PWR spectra can be described by three
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**TABLE I**

Binding constants for the interaction between G-proteins and the hDOR either unbound or prebound to agonist or antagonist

| Bound ligand | Agonist (DPDPE) | Antagonist (NTI) | No ligand |
|--------------|-----------------|-----------------|-----------|
| Polarization|                 |                 |           |
| $K_{D_{polarization}}$ (nM) | $p$ | $s$ | $p$ | $s$ | $p$ | $s$ |
| $K_{D_{GTP\gammaS}}$ (nM) | $p$ | $s$ | $p$ | $s$ | $p$ | $s$ |

* No PWR spectral shifts were obtained upon addition of GTPγS up to 1 μM.

**RESULTS**

Binding of G-proteins to Various Ligated States of the hDOR—From PWR spectral measurements such as those illustrated in Fig. 2 (A and B), it can be seen that bilayer deposition, agonist-bound receptor incorporation, and addition of a G-protein solution to the aqueous compartment of the PWR cell lead to increases in the resonance angle minimum and changes in the resonance depth for both $p$- and $s$-polarized light. These are related to increases of refractive index due to an increase in deposited mass on the resonator surface, as well as to increases in proteolipid film thickness (9, 22). To quantify these changes, it is necessary to carry out a full theoretical analysis of these spectra. Such analyses are presently underway and will be reported separately.

In these experiments, we do not directly determine the concentrations of receptor and G-protein in the PWR cell. Affinities are determined based on the PWR spectral changes that occur due to mass increases in the proteolipid system upon incremental addition of G-protein to the cell. Only material that is deposited on the resonator surface affects the PWR signal, i.e. there is no interference from the material that is in the bulk solution. Thus, the spectral changes are proportional to the amount of G-protein bound to the receptor and plots of spectral shifts versus bulk G-protein concentration allow a direct determination of binding affinity. In other words, each concentration point in a saturation curve corresponds to the total G-protein added to the aqueous compartment versus the amount bound, and it is assumed that the bulk material is able to freely diffuse and equilibrate with the membrane.

In the case of G-protein binding, control experiments (Fig. 2D) demonstrate that much smaller spectral shifts (<10%) occur when the same concentrations of G-protein solution as in Fig. 2 (A and B) are added to a bilayer that has not had hDOR incorporated. Plots of the G-protein concentration (final concentration in the cell compartment) versus the resonance position angular shift (Fig. 2C) yield a hyperbolic curve that can be fit to obtain the $K_d$ value for G-protein binding to the hDOR (Table I). In the absence of receptor, the smaller spectral shifts observed do not follow a hyperbolic curve over the concentration range used and thus are non-saturating (Fig. 2D); presumably, these correspond to nonspecific G-protein binding to the lipid bilayer. The $K_d$ values obtained for DPDPE-ligated hDOR are in good agreement with the previously determined $EC_{50}$ for DPDPE (19.1 ± 7.2 nM) obtained from [35S]GTPγS membrane assays (23). We should also point out that the total shifts in the PWR spectra in the presence of hDOR were larger for $p$-polarization than for $s$-polarization (~50 millidegrees for $p$-polarization versus ~40 millidegrees for $s$-polarization), which is characteristic of an anisotropic structural change, as was also observed previously for agonist binding to the hDOR (13, 15). This is consistent with the cylindrical shape of the receptor-G-protein complex. To our knowledge, the only other direct observations of G-protein binding to a GPCR have been carried out using surface plasmon resonance measurements of the rhodopsin/transducin system (17, 21, 24).

Fig. 2 (E and F) shows the effect of adding GTPγS (the non-hydrolyzable form of GTP) to the DPDPE-hDOR-G-protein complex. This caused a decrease in the incident angle of the resonance, which can be approximated by a lowering of the refractive index resulting from a decrease in mass in the proteolipid system (if the membrane thickness had changed, this should have produced a change in resonance amplitude as well (22), which was not observed). The angular shifts also followed a hyperbolic binding curve (Fig. 2G) with $K_v$ values as given in Table I. It is known that the receptor-activated exchange of GDP by GTP in the α subunit of the G-protein causes release of this subunit from the receptor and from the βγ subunit. Thus, if the α-subunit leaves the membrane entirely in the present system, the decrease in the membrane-bound mass in the proteolipid system could correspond to this process. However, it is known that the G-protein subunits are palmitoylated, myristoylated, and prenylated, and thus the α-subunit may remain membrane-bound even after activation. A second possibility is...
a change in the structure of the receptor complex that results in displacement of lipid from the bilayer into the Gibbs border that anchors the bilayer to the Teflon spacer separating the resonator from the aqueous compartment, as we have previously observed for agonist binding to the hDOR (13, 15). Further studies are required to clarify this point. GTP binding to transducin was also observed in the earlier surface plasmon resonance studies of rhodopsin (17, 21, 24). Because our previous work (13, 15)2 has shown that high affinity ligand binding to the hDOR occurs under the conditions of the present experiments, the fact that we can now also bind G-proteins with high affinity shows that the receptor incorporates into the lipid bilayer in a bidirectional manner, i.e. some of the molecules insert with their ligand binding site facing the external medium and others with their G-protein binding site facing in that direction. In Fig. 2 (H and I) we show the time courses for G-protein binding to the receptor (pre-bound with agonist) and for the spectral shifts, possibly due to a-subunit dissociation, following GTPγS addition. By fitting the data to an exponential function (solid lines), we have obtained the following rate constants: for G-protein binding to the receptor (pre-bound with agonist) and for the spectral shifts, possibly due to a-subunit dissociation, following GTPγS addition. The hDOR concentration was 4 nM and the G-protein concentration was 800 nM, the latter corresponding to the amount needed to reach saturation. C and D, PWR spectra for p- and s-polarization, respectively, obtained with the TMT-L-Tic-hDOR complex before (○) and after (●) addition of G-proteins (the receptor concentration in the sample cell was 4 nM and the G-protein concentration was 800 nM).

Applying the same strategy, we have studied the binding of G-proteins to hDOR pre-bound with the antagonist naltrindole (NTI) and have found that the binding to the receptor still occurred but with a 50-fold lower affinity (Table I). As in the case of the agonist-bound state, we found the process to be anisotropic with shifts in the p-polarization resonance of ~40 and ~30 millidegrees for the s-polarization resonance and to follow a hyperbolic saturation curve (Fig. 3A). The difference in G-protein binding affinity between agonist-bound and antagonist-bound receptor states is in agreement with studies using fluorescence spectroscopy, which support the existence of conformational heterogeneity of G-protein-coupled receptors, depending on the nature of the ligand being bound (25–27), as well as with recent studies of GPCRs using PWR, which have directly shown that an antagonist places the receptor into a different conformation from that produced by an agonist or inverse agonist (13, 15).2,3 It is also well known from pharmacological studies that antagonist binding produces conformations that are not favorable for G-protein binding and activation. Consistent with these observations is the fact that GTPγS addition to the antagonist-ligated hDOR-G-protein complex produced no additional PWR spectral shifts (Fig. 4, A and B). It is known that point mutations in receptors (for example, mutations of Phe-303 in the αβ-adrenoreceptor) can preserve high affinity for G-proteins but eliminate the ability of receptor agonists to produce G-protein activation (28). This suggests that the receptor conformations for binding to G-protein and for its activation may be distinct. It is also worth noting that the

2 I. Alves, S. Cowell, Z. Salamon, S. Devanathan, G. Tollin, and V. J. Hruby, unpublished data.

3 S. Devanathan, Z. Yao, Z. Salamon, B. Kobilka, and G. Tollin, unpublished data.
existence of antagonist-receptor-G-protein complexes has been observed for the μ-opioid receptor using the GTPγS assay (29).

The addition of G-proteins to the inverse agonist (TMT-L-Tic)-bound receptor was found to give even more pronounced differences than those obtained with antagonist. Thus, the G-protein does not recognize the receptor when in the inverse agonist-bound state (Fig. 4, C and D), as evidenced by the fact that the small shifts in the PWR spectra obtained here were indistinguishable from the nonspecific binding of the G-protein to the lipid bilayer that was observed in our control experiments (see above). This is consistent with the well known elimination of the basal activity of GPCRs (i.e. G-protein activation in the absence of agonist binding) by inverse agonists.

Again, as expected, addition of GTPγS produced no spectral changes (not shown).

We also have studied G-protein binding to a receptor in the unliganded state and have found that binding indeed occurs, although with a lower affinity than that observed for the agonist-bound state (Table I). Again, the process is anisotropic but to a lesser extent than for the agonist or antagonist-bound state, and the total shifts also were smaller (−30 millidegrees for p-polarization and −26 millidegrees for s-polarization, as seen in Fig. 3B), suggesting structural differences in the states produced. Constitutive activity has become a well described characteristic of many GPCRs and has helped to redefine the concept of how they function. Such ligand-independent activity
has been described for the opioid receptors, either in their wild-type form or in mutated forms (30). Even though it has long been thought that the inactivated receptor should be able to interact with G-proteins, and this state has been proposed by Kenakin as a component of the cubic ternary complex model of GPCR function (31, 32), up until now there has been no direct evidence of the existence of that state. The present studies directly demonstrate that the unliganded receptor corresponds to a distinct state from that of agonist, antagonist, and inverse agonist-bound species, because it binds to the G-protein with a different affinity and produces a different structure. Previous studies with the β2-adrenergic receptor point to this same conclusion based on thermal denaturation and proteolysis experiments (33, 34). It is important to point out, however, that, as in the antagonist and inverse agonist cases, we have observed no changes in the PWR spectra upon addition of GTPγS to the receptor-G-protein complex, suggesting that the unliganded receptor may not allow activation. This interesting observation requires further study.

**Binding of Individual G-protein Subtypes to Unliganded and Agonist-bound hDOR—**We have also investigated the interaction of the individual G-protein subtypes with the unliganded and DPDPE-bound states of the receptor. As an illustration of the results obtained in these experiments, Fig. 5 (A and B) presents the PWR spectra obtained for the interaction of the Go, plus βγ subunits (1:1 ratio) with the agonist-bound receptor. It should be pointed out that the total shift obtained upon G-protein binding to the receptor was comparable to the shifts obtained with the G-protein mixture, as expected because approximately the same amount of receptor was present in both cases. As can be seen in Fig. 5 (B and C) and Table II, the Kd values obtained for the binding of the Go, plus βγ subunits to the agonist-bound receptor and the GTPγS affinity for the receptor-G-protein complex are comparable to those obtained for the G-protein mixture.

Table II also presents a summary of the results obtained with the other G-protein subtypes. It is very interesting to note the high level of specificity in the binding of the G-protein subunits to the receptor, both in the unliganded and the agonist-bound states. The agonist-bound receptor has the highest affinity for the Go, and the Go subunits (−7 and 10 nt, respectively; slightly higher than the G-protein mixture), intermediate affinity for the Go subunit, and lowest affinity for the Go subunit. The unliganded receptor also has the highest affinity for the Go subtype (−20 nt), whereas it has intermediate affinity for the Go (−80 nt, higher than the agonist-bound state) and the Go subtypes, and, in contrast to the agonist-bound state, it has the lowest affinity for the Go subtype. Previous coimmunoprecipitation studies of the mouse δ-opioid receptor-G-protein complexes performed with antisera directed against different Go and Gβ subunits noted changes in the G-protein subtype association upon agonist stimulation (35). Thus, agonist binding causes an increase in association with Go and a decrease in association with Goα, dissociation of Goα and association with Goβ, and no change in association with Goβ. These results correlate with ours.

As can be seen in Table II, the affinities of GTPγS toward the receptor-G-protein complex are also very much dependent on the G-protein subtype that is bound, as well as on the receptor state. The highest affinity was found for the receptor-Goα1 complex (−4 nt) and the lowest for the receptor-Goα complex (−400 nt). In contrast to the agonist-bound state of the receptor, no PWR spectral shifts were observed upon addition of GTPγS (concentrations up to 5 μM), except for the receptor-Goα complex. It is also interesting to note that the GTPγS affinities do not track the G-protein affinities, adding another level of selectivity to these interactions. Previous GTPase activity studies done with the hDOR also observed that the Goα-bound receptor promoted greater GTP exchange than the Goα subtype, activating about three times more efficiently (36). Also of interest, in a recent study the dopamine receptor was expressed with four different G-protein subtypes (the same as used in the present work), and the coupling between receptor and G-protein was investigated upon agonist treatment (37). A high level of selectivity in the receptor-G-protein interaction was also found, as well as differential agonist activation of the four G-protein subtypes.

**DISCUSSION**

The present work has demonstrated that PWR spectroscopy can provide important new insights into membrane signaling by GPCRs. The results are consistent with the formation of distinct conformational states of the hDOR by binding of various types of ligand that interact differently with G-proteins and that correlate well with the known pharmacological activities of these different ligand classes. We are presently carrying out a more detailed analysis of the data described above, including theoretical fitting of the PWR spectra to evaluate refractive indices and thickness parameters. This will provide greater insights into the structural conformations of the various states produced in these experiments and will be reported separately.

The results obtained with the individual G-protein subunits reveal a high degree of diversity and selectivity that may be important in controlling the specific interactions of these receptors with multiple cellular effector systems. We are currently applying the same strategy to study receptor-G-protein interactions upon different agonist treatments. This should provide new insights into the basis for differential physiological and/or pharmacological effects of drug activity. It is important to point out that PWR spectroscopy allows one to pursue a great variety of studies without having to rely on labeling protocols, as well as avoiding more complicated and time-consuming techniques. We also note that it should also be possible to design experiments to elucidate events occurring further downstream in the signal transduction process, such as receptor down-regulation. As a consequence, the methods described here will allow new light to be shed on the pathways of signal transduction and should be extremely useful in drug discovery protocols.

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Sphingosylphosphorylcholine and lysophosphatidylcholine are ligands for the G protein-coupled receptor GPR4.

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This paper has been withdrawn.

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Direct observation of G-protein binding to the human δ-opioid receptor using plasmon-waveguide resonance spectroscopy.

Isabel D. Alves, Zdzislaw Salamon, Eva Varga, Henry I. Yamamura, Gordon Tollin, and Victor J. Hruby

PAGE 48891:

In the right column, under the heading "Lipid Bilayer Formation, hDOR Incorporation, and G-protein Addition," “methanol (0.05:0.95:0.5, v/v)” should read “methanol (0.05:9.5:0.5, v/v).”