Selectivity, Cooperativity, and Reciprocity in the Interactions between the δ-Opioid Receptor, Its Ligands, and G-proteins

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A better understanding of signal transduction mechanisms is of critical importance. Methodologies that allow studies to be done while receptors are incorporated into lipid bilayers are advantageous. One such technique is plasmon-waveguide resonance (PWR) spectroscopy, which can follow changes in conformation accompanying protein-ligand, protein-protein, and protein-lipid interactions occurring in G-protein-coupled receptors in real time with high sensitivity and without the need for molecular labeling. Here we investigated several aspects of human δ-opioid receptor (hDOR)-G-protein interactions: (1) the effect of different types of agonists on the interaction with individual G-protein subtypes; (2) the affinities of the separate G-protein α and βγ subunits to different ligand-occupied states of the receptor; and (3) the effect of the presence of the G-protein on the interactions of the ligand with the receptor. To accomplish this we have incorporated the receptor into a solid supported lipid bilayer in the presence of ligand or G-protein and monitored the PWR spectral changes induced by the reciprocal G-protein or ligand interactions. We found a high degree of selectivity in the interactions of different agonist-bound states of the receptor with the different G-protein subtypes. This has important implications for agonist-directed trafficking and selective drug design. Studies with the separated α and βγ subunits show that cooperativity exists in these interactions. The high affinities of the separated subunits to the receptor point to the possibility of independent promotion of specific signaling events. The presence of G-proteins increased the affinity of agonists to the hDOR, and caused faster binding kinetics and different ligand-induced conformational changes. Because ligand also influences G-protein binding, reciprocity exists between these two binding processes.

G-protein-coupled receptors (GPCRs)1 represent one of the largest families of proteins in mammals, with 5% of the total cell proteins belonging to this group. Together they constitute one of the principal targets of drugs used in pharmacology, especially in the central nervous system. A better understanding of the mechanisms of activation and signal transduction of these proteins seems imperative. Despite their crucial importance, however, very little is known about their structure/function relationships, mainly as a consequence of their low natural abundance and their membrane nature. The opioid receptors belong to this superfamily and consist of three receptor types: δ, μ, and κ (1). These receptors transmit the signals induced by binding of opioid peptides and opiate alkaloids such as morphine and are involved in a plethora of biological events such as stress-induced analgesia, locomotive activity, blood pressure, gastrointestinal motility, learning, and memory.

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 Gi, Go family of G-proteins (2–4). Such studies have usually been done using radiolabel assays, in which those interactions are monitored through GTPγS or CAMP assays that measure a downstream effect of the interaction and, most importantly, a response that comes from a mixture of G-protein subtypes. If one wants to monitor the interactions of a receptor with each G-protein subtype separately, one has to either do immunoprecipitation studies with an antibody against each subtype, to fluorescently label each subtype for optical spectroscopic studies, or to prepare different cell lines, each expressing solely one subtype of G-protein together with the receptor of interest. These methodologies in addition to being complex, time-consuming, and relying on the presence of labels, either fluorescent or radioactive, also suffer from the drawback of not providing complete information about these interactions, such as individual affinity constants and the nature of structural changes induced by binding.

In our laboratories we have been using plasmon-waveguide resonance (PWR) spectroscopy to investigate the conformational changes occurring upon ligand and G-protein binding to the human δ-opioid receptor (hDOR) incorporated into a solid-supported lipid bilayer (5–7). Because of its ability to obtain resonances with light polarized both perpendicular (p-polarization) and parallel (s-polarization) to the resonator surface, PWR can follow changes in conformation accompanying protein-ligand, protein-protein, and protein-lipid interactions occurring in G-protein-coupled receptors in real time with high sensitivity and without the need for molecular labeling. Here we investigated several aspects of human δ-opioid receptor (hDOR)-G-protein interactions: (1) the effect of different types of agonists on the interaction with individual G-protein subtypes; (2) the affinities of the separate G-protein α and βγ subunits to different ligand-occupied states of the receptor; and (3) the effect of the presence of the G-protein on the interactions of the ligand with the receptor. To accomplish this we have incorporated the receptor into a solid supported lipid bilayer in the presence of ligand or G-protein and monitored the PWR spectral changes induced by the reciprocal G-protein or ligand interactions. We found a high degree of selectivity in the interactions of different agonist-bound states of the receptor with the different G-protein subtypes. This has important implications for agonist-directed trafficking and selective drug design. Studies with the separated α and βγ subunits show that cooperativity exists in these interactions. The high affinities of the separated subunits to the receptor point to the possibility of independent promotion of specific signaling events. The presence of G-proteins increased the affinity of agonists to the hDOR, and caused faster binding kinetics and different ligand-induced conformational changes. Because ligand also influences G-protein binding, reciprocity exists between these two binding processes.

1 The abbreviations used are: GPCR, G-protein-coupled receptor; hDOR, human δ-opioid receptor; PWR, plasmon-waveguide resonance; GTPγS, guanosine triphosphate γS; DPDPE, c-[d-Pen2,d-Pen5]enkephalin; NTI, naltrindole; TMT-L-Tic, (2S,3R)-β-methyl-2,6-dimethyl-tyrosyltetrahydroisouquinoline-3-carboxylic acid; mdeg, millidegree.

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CURRENTLY CURATING IN GPCRs IN REAL TIME WITH HIGH SENSITIVITY AND WITHOUT THE NEED FOR MOLECULAR LABELING. IN THE PRESENT STUDIES WE HAVE USED PWR TO OBTAIN A BETTER UNDERSTANDING OF SEVERAL ASPECTS OF G-PROTEIN INTERACTION WITH THE hDOR. THEREFORE, WE HAVE SHOWN THAT DIFFERENT AGONIST-BOUND STATES OF THE hDOR HAVE DISTINCT AND SELECTIVE INTERACTIONS WITH DIFFERENT G-PROTEIN SUBTYPES. IN ADDITION, WE HAVE FOUND THAT THE SEPARATED α AND βγ G-PROTEIN SUBUNITS ARE TIGHTLY BOUND TO THE hDOR, AND THAT THE ACTIVATED RECEPTOR CAN STIMULATE GDP/GTP EXCHANGE IN THE α SUBUNIT IN THE ABSENCE OF THE βγ SUBUNIT. FINALLY, WE HAVE DEMONSTRATED THAT THE PRESENCE OF THE G-PROTEIN AFFECTS LIGAND BINDING AND CONFORMATIONAL CHANGES IN THE hDOR. WE BELIEVE THESE STUDIES PROVIDE NEW INSIGHTS INTO THE FIRST PART OF THE SIGNAL TRANSDUCTION PATHWAY FOR THE hDOR. THEY SHOULD ALSO BE IMPORTANT FOR RATIONAL DRUG DESIGN PROTOCOLS WHEREBY LIGANDS COULD BE DESIGNED THAT TARGET SPECIFIC PATHWAYS, IN THIS WAY AVOIDING THE KNOWN DETRIMENTAL SIDE EFFECTS OF CERTAIN DRUGS.

EXPERIMENTAL PROCEDURES

Receptor Purification and Characterization—A fully functional receptor, labeled at the C terminus with a myc epitope and His tag, was stably transfected into a Chinese hamster ovary cell line (CHO-K1) (8). The receptor was purified by metal chelating and ligand affinity chromatography and characterized as published previously (6, 8). For the studies in which the receptor was pre-bound with ligand, the hDOR was preincubated with saturating amounts (at least 1 order of magnitude higher concentration than published binding affinities) of ligand, using two peptide agonists, DPDPDE (American Peptide Co.) and deltorphin II (prepared in our laboratory by standard methods of solid phase peptide synthesis (9)), two non-peptide agonists, SNC80 (Tocris Inc., Elsilive, MO) and (−)T6-N7 (Toray Industries, Kamakura, Japan), a partial agonist, morphine (Sigma), an antagonist, NTI (Sigma), and an inverse agonist, TMT-L-Tic (synthesized in Dr. Hruby’s laboratory following published procedures (10)). The receptor was incubated with the respective concentrations of ligand prior to incorporation into the PWR cell that contained a previously deposited lipid bilayer on the resonator surface.

The concentration of the solubilized receptor was determined using a BCA assay (Pierce). The purple reaction product was monitored at 560 nm using an enzyme-linked immunosorbent assay plate reader (µQuant, Bio-Tek Instruments, Inc.). Following purification, the quality of the receptor protein was assessed by determining the specific activity, i.e. the number of functional receptors per mol of receptor (ligand binding) per mol of receptor protein. This was performed by introducing the detergent-solubilized hDOR (pre-bound with the ligand to be investigated) into the aqueous compartment under conditions that dilute the detergent to below the critical micelle concentration, which allows the membrane protein to incorporate spontaneously into the lipid bilayer. In order to make sure that all receptor molecules had ligand bound before adding the receptor to the PWR cell sample, we have added that same ligand to the sample compartment at a concentration above the KD value for that ligand. In these experiments we only have access to the side of the lipid bilayer facing the aqueous compartment (i.e. no direct access is available to the side of the lipid bilayer facing the silica surface of the prism), and we think that neither the G-protein nor most ligands are able to cross the bilayer. However, because binding to both the G-proteins and ligands can occur after receptor incorporation (and those processes are known to occur in opposite faces of the receptor), it appears that the receptor inserts spontaneously into the lipid bilayer, i.e. with the extracellular side facing both the silica surface and the aqueous compartment. Because we were interested in studying the ternary complex, i.e. ligand, receptor, G-protein, we have accomplished this, as we have done previously (6), by pre-binding 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 phase. 

Addition—In order to investigate the influence of G-protein binding on ligand interactions with the hDOR, the following procedure was used. Large unilamellar vesicles (liposomes), with the same lipid composition as the bilayer, containing incorporated receptor were prepared by mixing the solubilized and purified receptor (in a mole ratio of ~500:1, with or without the presence of the G-proteins (hDOR/G-protein in a 1:1 mole ratio) in 10 mM Tris buffer, pH 7.4, containing 100 mM KCl, followed by 10 freeze/thaw cycles and extrusion using polycarbonate membranes (200-nm pore size). Fusion of liposomes with the bilayer was then induced by the presence of calcium in the same buffer in the PWR sample cell compartment, and the resulting bilayer was monitored. This was achieved by varying the angle, ϕ, at a fixed λ (543.5 nm excitation was used). 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. 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 uniaxially oriented anisotropic systems such as biomembranes containing integral proteins (15–17).

Under the experimental conditions employed in this work, the optical changes were recorded with parallel and perpendicular polarization reference direction, and those obtained with s-polarization refer to the parallel direction, relative to the bilayer membrane surface. 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). For nonpherical molecules oriented...
uniaxially on the resonator surface, which is the case with GPCRs, and with G-proteins, the molecules deposited on the resonator surface are not absorbed at the excitation wavelength, and thus only and values influenced the PWR spectra. These parameters are determined by the surface mass density and the mass distribution (orientation and conformation) of the deposited materials. We have used the angular position of the resonance as the primary indicator of the surface mass density and the mass distribution on the surface. It should be pointed out that in these experiments, we do not know the ratio of to the molecule perpendicular to the membrane plane. Such resonance shifts are a consequence of changes in mass resulting from the deposition and interactions of molecules of high molecular weight such as the receptor and G-proteins. Changes in the resonance depth of the spectra are also correlated with alterations in the thickness and refractive index of the proteolipid film, again as a consequence of material deposition and distribution on the surface. It should be pointed out that the spectral changes observed upon lipid bilayer deposition and receptor incorporation into the bilayer are highly anisotropic (i.e. the changes in -polarization are quite different from those for -polarization) due to the highly anisotropic optical properties of oriented lipid bilayers and GPCRs. Previous results obtained upon incorporation of the into a lipid bilayer (5) have shown by theoretical fitting to the PWR spectra (12) that the thickness of the proteolipid system increased from 5.3 nm in the absence of receptor to 6.8 nm after receptor incorporation (the latter value corresponds to the dimension of the incorporated protein molecule perpendicular to the membrane plane). This value for the thickness of the proteolipid layer after receptor incorporation correlates well with the size determined for rhodopsin from x-ray crystallography (18). Spectral shifts obtained upon receptor incorporation were larger for -polarization than for -polarization (indicating refractive index changes in the direction that were larger than for the s-direction), which is a consequence of the anisotropic structure (i.e. cylindrical shape) of the receptor molecules. This is also evident for the uniaxial incorporation of the receptor into the bilayer with the expected orientation (i.e. long axis oriented perpendicular to the lipid bilayer), rather than just randomly adsorbed to the bilayer surface, 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 with both ligands (5, 7) and with G-proteins (6), we presume that the receptor is incorporated bi-directionally 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. We do not, however, know the ratio between these two orientations.

It should also be noted that in these experiments, we do not

**RESULTS AND DISCUSSION**

**Interaction of G-protein Subtypes with Different Agonist-bound States of the hDOR—**Fig. 1, A and B, shows typical results from the PWR spectral measurements carried out in the present experiments, illustrated using the partial agonist morphine. Similar experiments have also been performed by using other ligands (see below). As can be seen, bilayer deposition, receptor incorporation, and addition of a G-protein solution to the aqueous compartment of the PWR cell lead to increases in the position of the resonance angle minimum and changes in the resonance depth for both - and -polarized exciting light. Increases in the resonance angle position are related to increases in the refractive index and thickness of the material deposited directly onto the resonator surface. Such resonance shifts are a consequence of increases in mass resulting from the deposition and interactions of molecules of high molecular weight such as the receptor and G-proteins. Changes in the resonance depth of the spectra are also correlated with alterations in the thickness and refractive index of the proteolipid film, again as a consequence of material deposition and distribution on the surface. It should be pointed out that the spectral changes observed upon lipid bilayer deposition and receptor incorporation into the bilayer are highly anisotropic (i.e. the changes in -polarization are quite different from those for -polarization) due to the highly anisotropic optical properties of oriented lipid bilayers and GPCRs. Previous results obtained upon incorporation of the into a lipid bilayer (5) have shown by theoretical fits to the PWR spectra (12) that the thickness of the proteolipid system increased from 5.3 nm in the absence of receptor to 6.8 nm after receptor incorporation (the latter value corresponds to the dimension of the incorporated protein molecule perpendicular to the membrane plane). This value for the thickness of the proteolipid layer after receptor incorporation correlates well with the size determined for rhodopsin from x-ray crystallography (18). Spectral shifts obtained upon receptor incorporation were larger for -polarization than for -polarization (indicating refractive index changes in the direction that were larger than for the s-direction), which is a consequence of the anisotropic structure (i.e. cylindrical shape) of the receptor molecules. This is also evident for the uniaxial incorporation of the receptor into the bilayer with the expected orientation (i.e. long axis oriented perpendicular to the lipid bilayer), rather than just randomly adsorbed to the bilayer surface, 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 with both ligands (5, 7) and with G-proteins (6), we presume that the receptor is incorporated bi-directionally 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. We do not, however, know the ratio between these two orientations.

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Ligand and G-protein Interactions with the DOR

Table I

| G-protein subtype | G_{α1} | G_{α2} | G_{α3} |
|------------------|--------|--------|--------|
| DPDPE            |        |        |        |
| $K_D^{G-protein}$ (nM) | 10 ± 1 | 304 ± 26 | 7 ± 1 |
| $K_D^{GTP-γS}$ (nM)  | 402 ± 54 | 4.2 ± 0.5 | 9.1 ± 0.7 |
| Deltorphin II     | 4 ± 0.3 | 58 ± 8 | 6 ± 0.8 |
| $K_D^{G-protein}$ | 11 ± 1.2 | 92 ± 11 | 7.5 ± 0.6 |
| $K_D^{GTP-γS}$ (nM)  | 5.0 ± 0.5 | 215 ± 33 | 13.6 ± 1.1 |
| (--)-Tan 67       | 12 ± 1.2 | 23 ± 1.7 | 186 ± 21 |
| $K_D^{G-protein}$ | 8.5 ± 0.7 | 75 ± 9 | 13 ± 1.1 |
| $K_D^{GTP-γS}$ (nM)  | 9.5 ± 1.8 | 2.3 ± 0.4 | 96 ± 14 |
| Morphine          | 43 ± 5 | 34 ± 8 | 310 ± 27 |
| $K_D^{G-protein}$ | 895 ± 128 | 93 ± 10 | 1650 ± 135 |
| $K_D^{GTP-γS}$ (nM)  | 310 ± 27 | 17 ± 3 | 915 ± 145 |

$K_D$ values for Ga subtypes (in the presence of a mixture of βγ dimers) and hDOR bound to various ligands and between GTP-γS and the ligand-receptor-G-protein complex.

$K_D$ values were obtained from plotting the resonance minimum position for the PWR spectra as a function of G-protein/GTP-γS concentration and fitting to the following hyperbolic function that describes the binding of a ligand to a receptor: $y = \frac{B_{max} 	imes x}{K_D + x}$, $B_{max}$ represents the maximum concentration bound, and $K_D$ is the concentration of ligand required to reach half-maximal binding. Results presented were obtained from three independent experiments, and standard deviations are presented. No PWR spectral shifts were obtained upon addition of GTP-γS up to 5 μM.

| Ligand and G-protein Interactions with the DOR |
|-----------------------------------------------|

$G_{α1}$, $G_{α2}$, and $G_{α3}$ subunits; the SNC80-bound receptor has the highest affinity for the Gα3 subunit, the (--)Tan67-bound receptor has the highest affinity for the Gα1 subunit, and the (--)Tan67-bound receptor has highest affinity for the Gα3 subunit. Furthermore, these affinity differences are quite large, as much as 50-fold in some cases. Perhaps the most interesting were the differences observed with GTP-γS interactions, with affinities varying by as much as 100-fold in some cases. It is also important to note that the capability of the bound receptor to bind the G-protein and the ability of the G-protein to undergo GTP exchange were shown.
to be separate phenomena. For example, the morphine-bound receptor binds most strongly to the \( \text{G}_\alpha \text{o}_\text{S} \) subtype, but the highest affinity to \( \text{GTP}_\text{S} \) is to the \( \text{G}_\alpha \text{r} \) complex. Similarly, the relative affinities for the other \( \text{G}_\alpha \) subtypes and \( \text{GTP}_\text{S} \) are uncorrelated. As is evident from Table I, the other ligand-bound receptors display analogous behavior. This is a very important finding that could not be obtained by the classical pharmacological assays to measure drug activity (e.g. cAMP and \( \text{GTP}_\text{S} \) radiolabel assays). It provides a direct experimental basis and quantification for the phenomenon of agonist-directed drug trafficking, well known in pharmacology (20), and provides a new basis for the drug design of ligands that will utilize specific downstream pathways.

When comparing the results obtained with the full agonists DPDPE, deltorphin II, (-)-Tan67, and SNC80 and those with the partial agonist morphine (Table I), one can see that although the affinities for the different \( \text{G}_\alpha \) protein subtypes are mostly high for all the ligands, the affinities of \( \text{GTP}_\text{S} \) for the receptor-\( \text{G}_\alpha \)-protein complex are markedly lower in almost all cases for the partial agonist than for the full agonists. This demonstrates that, at least for the hDOR, partial agonism is correlated with a reduced ability of the \( \text{G}_\alpha \)-protein to promote GDP/GTP exchange. This result again could only have been quantified by direct measurements of \( K_D \) values, as opposed to indirect pharmacological assays.

The lower affinity of \( \text{GTP}_\text{S} \) to the receptor when bound with a partial agonist may be a consequence of the receptor being in a different conformational state than that observed when agonist is bound. Furthermore, the differences observed here between peptide and non-peptide agonists also seems to have a similar basis. Thus, previous studies from our laboratories involving the hDOR (5, 7) and from our laboratory (21) and other laboratories (22, 23) involving the \( \beta_\text{ad} \)-adrenergic receptor clearly demonstrate that these various types of ligands produce different receptor conformations with distinct kinetic behaviors. Moreover, theoretical studies by Kenakin (24, 25) have predicted that GPCRs can adopt multiple conformations upon receptor occupation by different ligands leading to different functional properties. The selectivity observed here in the interactions with the four \( \text{G}_\alpha \) protein subtypes upon differential agonist activation of the receptor generates another level of multiplicity in terms of GPCR signaling, with certain pathways being preferentially selected over others thus giving rise to a larger diversity in terms of cellular responses. This opens the possibility of using PWR methods for selective drug design.

**Individual Interactions of the \( \text{G}_\text{a} \) Subunit and the \( \text{G}_\beta\gamma \) Dimer with Different Agonist-bound States of the hDOR**—It has long been accepted that the first signaling event in GPCRs involves their interaction with \( \text{G}_\text{a} \)-proteins (26), which so far has mainly been studied in their trimeric form. Here we have investigated the separate interactions of the \( \alpha \) and \( \beta\gamma \) subunits with the hDOR when pre-bound with different types of ligands. In these studies we have chosen the \( \text{G}_\alpha \) protein subunit with the highest affinity for each ligand-bound state of the receptor (see Table I). For example, in the case of DPDPE we have studied the interaction of the \( \text{G}_\alpha \text{a}_\text{S} \) subunit (in the absence of \( \text{G}_\beta\gamma \)) and that of \( \text{G}_\beta\gamma \) (using the mixture of subtypes found in brain) in the absence of \( \text{G}_\alpha \text{r} \). As shown in Fig. 2, A and B, and Fig. 3, A and B, for the case of DPDPE, the PWR resonance angle position increased as the \( \text{G}_\alpha \) subunit was added to the proteolipid system (for both the \( \text{G}_\text{a}_\text{S} \) and the \( \text{G}_\beta\gamma \) subunits). This agrees with results presented in the previous section and can be ascribed mainly to an increase in mass. By plotting the PWR resonance position versus the \( \text{G}_\alpha \) subunit concentrations (Fig. 2C and Fig. 3C), dissociation constants were obtained for each subunit interaction with the receptor; these are presented in Table II. One can see that the affinity of the \( \text{G}_\alpha \text{a}_\text{S} \) subunit was greatly decreased (\( K_D \) values increased from ~7 to ~200 nM) when the \( \beta\gamma \) dimer was absent, whereas the affinity of the \( \beta\gamma \) dimer to the receptor was decreased to a smaller extent (\( K_D \) increased to ~40 nM) when in the absence of the \( \text{G}_\alpha \text{a}_\text{S} \) subunit. It should also be noted that the binding of the two subunits to the receptor resulted in very different anisotropic structural...
changes, illustrated by the differences in the relative magnitudes of the s- and p-polarized shifts (Fig. 2C and Fig. 3C). The precise reason for this is not clear at present, but it may indicate the formation of different conformations of the proteolipid system when one or the other of the subunits is bound, or it may just be a consequence of the different structures of those subunits. Because PWR is sensitive to changes in the optical properties of all components present in the proteolipid system, the observed difference could arise from the lipid, the receptor, or the G-protein, or from any combination of these. We cannot distinguish these possibilities at present, although this could possibly be dissected by labeling each component with a different chromophore and using a laser with the appropriate wavelength to follow each component separately (15).

In order to investigate whether the decreased affinity was dependent on the identity of the Ga subunit used, we have done the same experiments with the other Ga subtypes, and we have found that the lower affinity observed for the G\textsubscript{a16G} subunit when in the absence of the \(\beta\gamma\) dimer was independent of the Ga subtype (data not shown). We have also investigated whether the smaller affinity change of the \(\beta\gamma\) dimer compared with the \(\alpha\) subunit (when separated) is modulated by the type of ligand that is bound to the receptor. As can be seen in Table II, and by comparing these values with the results presented in Table I, the \(\beta\gamma\) dimer always showed a smaller affinity change to the receptor than the \(\alpha\) subunit, independent of the ligand that was bound to the receptor. These results correlate well with published x-ray diffraction studies regarding rhodopsin-transducin interactions, where the presence of a distinct contact interface between the receptor and the \(\beta\gamma\) subunit was implied by the structure (27).

We have also investigated whether the \(\beta\gamma\) dimer is necessary for the catalytic activity of the \(\alpha\) subunit. As seen in Fig. 4, the addition of GTP\textsubscript{γS} to the DPPDE-hDOR-G\textsubscript{a16G} complex caused an appreciable decrease in the angular position of the resonance spectrum, similar to the results reported above for the entire G-protein heterotrimer. However, in this case the spectral position shifted completely to that of the DPPDE-hDOR complex with no G-protein present, suggesting that all of the G\textsubscript{a} subunit was dissociated from the proteolipid system upon GTP\textsubscript{γS} interaction. Furthermore, even though the presence of the G\(\beta\gamma\) subunit was not essential for the catalytic activity of the \(\alpha\) subunit, as seen by comparing results in Table I and Table II, the affinity of the GTP\textsubscript{γS} subunit to the receptor was decreased by 10–20-fold when the \(\beta\gamma\) subunit was absent. Similar results have been observed (28) in studies involving the rhodopsin-transducin interaction, in which the presence of the \(\beta\gamma\) subunit was also found not to be necessary for the interaction of the G\textsubscript{a} (the G\textsubscript{a} subunit of transducin) with the receptor nor for the GDP/GTP exchange, although its presence caused a 10-fold promotion of this process.

The present studies show that the presence of each G-protein subunit (\(\alpha\) subunit and \(\beta\gamma\) dimer) enhances the affinity of the other to the hDOR in a cooperative manner that is independent of the subtype of G-protein and of the ligand that is bound to the receptor. The high affinities obtained for the individual subunits to the agonist-activated receptor (especially for the \(\beta\gamma\) subunit) suggest that the separated subunits may also have a biological function. Such a role might be particularly relevant for tissues or cell compartments where some of the subunits are expressed to lesser extents, as has been shown to be the case for the hDOR (29). It is well known that the \(\alpha\) and \(\beta\gamma\) subunits of

![Graphs and data](https://example.com/graphs.png)

**Table II**

| G-protein subtype | DPDPE | G\textsubscript{a16G} | GTP\textsubscript{γS} | SNC80 | Morphine |
|-------------------|-------|-----------------|-------------|--------|----------|
| G\textsubscript{a}  |       |                 |             |        |          |
| K\textsubscript{i} | 213 ± 46 | 44 ± 5          | 282 ± 53   | 26 ± 3 | 352 ± 48 |
| K\textsubscript{i} | 90 ± 9  |                 | 156 ± 29   | a      | 910 ± 108 |

*No PWR spectral shifts were obtained.*
the G-protein interact with different effectors leading to different signal transduction events in the cell (30), and thus the capability for the subunits to act independently of one another may be essential for specific signaling pathways to be activated.

Ligand Interaction with the Low and High Affinity States of the Receptor—In Fig. 5, A and B, one can see that the fusion of liposomes containing the hDOR and G-proteins (the mixture of subtypes found in brain was used in these experiments) resulted in an increase in the resonance angle position. These shifts were highly anisotropic, with shifts in p-polarized resonances (130 mdeg) being larger than in s-polarized resonances.
no changes in the affinity of the ligand to the receptor were observed in the absence versus the presence of the G-protein (Table III). This demonstrates that only the agonist was able to generate a high affinity state of the receptor, as expected based on pharmacological experiments.

It is interesting to note that the PWR spectral changes obtained upon DPDPE binding to the unbound hDOR versus the hDOR-G-protein complex were quite distinct in terms of the magnitude of the spectral changes. Thus, as seen in Fig. 5, C and D, the addition of ligand to the hDOR (no G-protein present) caused spectral changes of considerably larger magnitude, especially for the p-polarized resonance (−25 mdeg versus −9 mdeg and 10 versus 7 mdeg for p- and s-polarization, respectively). The values obtained in the absence of the G-protein correlate well with previous results obtained with this receptor (7). This demonstrates that in the presence of the G-protein, the PWR spectral changes were much less anisotropic. This provides a direct indication that the structural changes induced in the proteolipid membrane by ligand binding in the presence and absence of G-protein were quite different. In particular, the larger magnitude of the p-polarized resonance change suggests that the perpendicular dimensions of the membrane were changed to a greater extent in the absence of the G-protein than in its presence. Thus, the G-protein apparently acts to constrain the movement of the transmembrane and extramembrane regions of the receptor and its associated lipid molecules.

The magnitudes of the PWR spectral shifts produced with the various ligands in the presence and absence of G-proteins are given in Table IV. From these values one can see that the p-polarized spectral changes upon ligand addition to the receptor in the presence of G-protein were uniformly smaller than in the absence of G-protein for all of the agonist ligands tested. In contrast, in the case of the antagonist (NTI) and the inverse agonist (TMT-L-Tic), there were little or no differences in the magnitudes of the PWR spectral shifts obtained upon ligand binding in the presence or absence of G-proteins. We suggest that this is a consequence of the receptor adopting a different conformation when occupied with antagonist and inverse agonist as compared with the agonist case, which locks the receptor into an unfavorable conformation for G-protein binding, so that the presence of the G-protein cannot modify the effect of the ligand on the receptor conformation. These results are consistent with the absence of an effect of the presence of the G-protein on the $K_d$ value for antagonist and inverse agonist binding, whereas large effects were observed for agonist binding (Table III). Previous PWR studies (7) have indeed shown that the antagonist and inverse agonist interaction with the hDOR induces different conformations in the receptor. Moreover, PWR studies involving measurements of the affinity of G-proteins to the receptor have shown that the antagonist-

| Table III | Table IV |
| --- | --- |
| **Ligands** | **Magnitude of the PWR spectral shifts obtained upon binding of ligand to the hDOR in the presence and absence of G-protein** (mixture of subtypes present in brain) |
| KD (nM) | p-polarization | s-polarization |
| KD (nM) | p-polarization | s-polarization |
| --- | --- | --- |
| **DPDPE** | 20 ± 5 | 2.5 ± 0.2 |
| **SNC80** | 54 ± 10 | 9 ± 2 |
| **Morphine** | 530 ± 30 | 357 ± 22 |
| **NTI** | 0.024 ± 0.001 | 0.023 ± 0.003 |
| **TMT-L-Tic** | 2.8 ± 0.3 | 3.0 ± 0.2 |

$^a$From Ref. 7.

$^b$No overall PWR spectral shifts were observed (cf. Ref. 7).
occupied hDOR has much lower affinity for G-proteins than the agonist-bound receptor (~50-fold), and that the inverse agonist-bound receptor does not interact with G-proteins up to micromolar concentrations (6).

The kinetics of the spectral changes occurring upon ligand binding to the hDOR was also quite different depending upon whether or not the G-protein was present (Fig. 6, A and B, and Table V). Thus, as reported previously (7), when the G-protein was absent conformational changes induced in the receptor by DPDPE binding were slow with at least one intermediate state of the receptor. Rate constants for this and other ligands are presented in Table V. Similar results were obtained in Ref. 8.

Although no direct evidence has ever been obtained. The present work provides such evidence. It should also be noted that, because the PWR signal is sensitive to structural changes occurring in the entire proteolipid system, the present experiments cannot distinguish between conformational changes occurring to varying extents in some combination of the hDOR, the G-protein, and the membrane lipids. Further experiments involving the use of labeled components and excitation wavelengths where those chromophores absorb will be necessary to obtain additional insight into this question.

Conclusions—The present work has demonstrated that PWR spectroscopy can provide important new insights into membrane signaling by GPCRs. 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. The fact that different ligands belonging to the same general class (i.e. agonists) lead to preferential interaction with certain G-protein subtypes has important implications for drug design. A tremendous amount of work has been done over the last half-century by scientists in search of ligands for the opioid and other receptor systems that suppress the detrimental side effects of these drugs while retaining the desired primary effects. This has been especially problematic for opiates such as morphine. One of the origins of such side effects is the lack of selectivity of those ligands regarding their target receptors, i.e. they can target different subtypes of the same receptor or even different GPCRs. Our results, demonstrating that different ligands from the same class can lead to preferential interaction with certain G-protein subtypes, suggest that these preferences may lead to an additional source of drug side effects. Thus, certain ligands, by selecting specific G-protein subtypes over others, may thereby be targeting specific signaling pathways that may lead to unwanted side effects. By designing ligands that activate only desired pathways, this might be avoided. PWR studies of the kind presented here may therefore have a great impact on drug screening protocols and design, as well as providing new insights into the basis for differential physiological and/or pharmacological effects of drug activity.

We have also shown that the simultaneous presence of individual G-protein subunits, α subunit and βγ dimer, enhances the affinity of the other to the hDOR in a cooperative manner. The high affinities obtained for the individual subunits to the agonist-activated receptor (especially for the βγ subunit) suggest that the separated subunits may also have a biological function. There is evidence that G-proteins are differentially expressed in different tissues (27). Furthermore, the α and β...
subunits of the G-protein interact with different effectors leading to different signal transduction events in the cell (28). Considering the above, the fact that the subunits have the capability to act independently, with the α subunit even maintaining its catalytic activity in the absence of the βγ subunit, may be essential for specific signaling pathways to be activated over others. Extensions of this work involve investigations of the interaction of the receptor with the different subtypes of the Gβ and Gγ subunits. At the present time, 5 different Gβ subunits and 12 different Gγ subunits are known (33). The present evidence suggests that specificity exists with respect to the influence of various Gβγ subtypes on a wide range of effectors, although not all combinations are found in all tissues. For example, the primary dimer in the brain is Gβ1γ2, whereas in the retina Gβ1γ1 predominates. Based on these findings, it is clear that ligand-specific activation of individual G-protein subtypes can produce distinct metabolic effects. Elucidation of these specificities can thus have useful consequences.

The experiments involving liposome fusion to a lipid bilayer demonstrate that one can use this strategy in designing PWR experiments in which one wishes to deliver molecules to the side of the lipid bilayer facing the prism surface that is otherwise inaccessible. Such experiments should be of great interest for studies of GPCR signal transduction involving downstream effectors such as adenyl cyclase or modulators such as kinases and arrestins. We have also directly observed that the affinity of the agonist DPDPE to the hDOR is modulated by the presence of the G-protein, its affinity being higher when G-protein is present (representing the so-called high affinity state) than when the G-protein is absent (i.e., the low affinity state of the receptor). Combining this with our earlier demonstration that ligand binding influences G-protein affinity (6), it appears that reciprocality exists in the functional properties of this GPCR. This must be a consequence of the ability of the receptor to transduce information across the lipid bilayer via conformational changes, a property that lies at the heart of GPCR function.

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Selectivity, Cooperativity, and Reciprocity in the Interactions between the δ-Opioid Receptor, Its Ligands, and G-proteins
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Selectivity, cooperativity, and reciprocity in the interactions between the δ-opioid receptor, its ligands, and G-proteins.

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In the left column, four lines from the bottom, “methanol (0.05:0.95:0.5, v/v)” should read “methanol (0.05:9.5:0.5, v/v).”