0026-895X/00/061132-10$3.00/0
MOLECULAR PHARMACOLOGY

MOL 57:1132–1141, 2000 /12867/824351

Reconstitution of the Human 5-HT_{1D} Receptor-G-Protein Coupling: Evidence for Constitutive Activity and Multiple Receptor Conformations

REGINALD BRYS,[1] KATTY JOSSON,[1] MARIA PAOLA CASTELLI, MIREK JURZAK, PETER LIJNEN, WALTER GOMMEREN, and JOSÉE E. LEYSEN

Janssen Research Foundation, Department of Biochemical Pharmacology, Beerse, Belgium (R.B., K.J., M.J., P.L., W.G., J.E.L.), University of Cagliari, Department of Neuroscience, Cagliari, Italy (M.P.C.)

Received July 19, 1999; accepted February 20, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

The 5-hydroxytryptamine (5-HT) 1D/1B receptors have gained particular interest as potential targets for treatment of migraine and depression. G-protein coupling and other intrinsic properties of the human 5-HT_{1D} receptor were studied using a baculovirus-based expression system in Sf9 cells. Coexpression of the human 5-HT_{1D} receptor with $G\alpha_{i1}$, α_{i2}, α_{i3}, or $G\alpha_o$-proteins and $G\beta_1\gamma_2$-subunits reconstituted a Gpp(NH)p-sensitive, high affinity binding of [^{3}H]5-HT to this receptor, whereas the $G\alpha_q\beta_1\gamma_2$ heterotrimer was ineffective in this respect. Competition of [^{3}H]5-HT binding by various compounds confirmed that coexpression of the human 5-HT_{1D} receptor with $G\alpha_{i/o}\beta_1\gamma_2$ reconstitutes the receptor in a high affinity agonist binding state, having the same pharmacological profile as the receptor expressed in mammalian cells. Binding of the antagonist ocaperidone to the human 5-HT_{1D} receptor in coupled or noncoupled state was analyzed. This compound competed with [^{3}H]5-HT binding more potently on the human 5-HT_{1D} receptor in the noncoupled state, showing its inverse agonistic character. Ocaperidone acted as a competitive inhibitor of [^{3}H]5-HT binding when tested with the coupled receptor form but not so when tested with the noncoupled receptor preparation. Finally, [^{35}S]GTPγS binding experiments using the inverse agonist ocaperidone revealed a high level of constitutive activity of the human 5-HT_{1D} receptor. Taken together, the reconstitution of the human 5-HT_{1D} receptor-G-protein coupling using baculovirus-infected Sf9 cells made possible the assessment of coupling specificity and the detection of different binding states of the receptor induced by G-protein coupling or ligand binding.

The neurotransmitter serotonin is involved in the modulation of a wide variety of physiological processes. 5-Hydroxytryptamine (5-HT) acts through a large group of receptors (13 subtypes of which have been identified to date) that were classified into seven distinct families according to their signaling properties and molecular structure (Hoyer and Martin, 1997). All these receptors, with the exception of the 5-HT_3 receptor (R) (a ligand-gated ion channel) are members of the G-protein-coupled receptor (GPCR) superfamily.

Two members of the 5-HT_1 family of serotonin receptors, i.e., the 5-HT_{1D}R and 5-HT_{1B}R, were given particular attention because of their apparent role in migraine and depression [see Moskowitz (1992); Halazy et al. (1997); and Pauwels (1997) for reviews]. Even if localization studies gave some indications about the respective physiological role of both receptors, the distinct role of the 5-HT_{1D}R and 5-HT_{1B}R often remains unclear. In peripheral tissues, only the 5-HT_{1B}R could be demonstrated where it mediates vasoconstriction (Hamel et al., 1993). In the central nervous system, the occurrence of the 5-HT_{1D}R is more restricted than that of the 5-HT_{1B}R (Bonaventure et al., 1997). However, in the trigeminal system (which is involved in neurogenic pain processes) the 5-HT_{1D}R mRNA is more abundant than the 5-HT_{1B}R mRNA (Bonaventure et al., 1998).

Despite the relatively low homology between both receptors at the amino acid level (63%), various ligands bind with similar potencies to both of them (Weinshank et al., 1992). Early 5-$HT_{1B/1D}$ receptor studies relied on nonselective compounds, which also acted as antagonists on several 5-HT_1 and 5-HT_2 receptor subtypes. Among these, ketanserin was shown to preferentially bind on the human (h) 5-HT_{1D} receptor (Peroutka, 1994; Wurch et al., 1998). Ocaperidone, an antagonist that has a higher affinity for h5-HT_{1D}R than for h5-HT_{1B}R, is also a 5-HT_{2A} and D_2 receptor antagonist (Leysen et al., 1992; Leysen et al., 1996; Lesage et al., 1998). The physiological functions that can specifically be attrib-

This work was supported in part by the IWT Grant 940232.
[1] These authors contributed equally to the work.

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; R, receptor; $G\alpha_{i/o}$, combination of $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_o$; GPCR, G-protein-coupled receptor; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); Sf9 cells, *Spodoptera frugiperda* cells; pIC_{50}, −log of the IC_{50} value; pEC_{50}, −log of the EC_{50}, value.

uted to the 5-HT$_{1B}$R and to the 5-HT$_{1D}$R could not be determined using these compounds because of their broad pharmacological profiles. The first selective h5-HT$_{1B}$/$_{1D}$R ligands described were a series of benzanilide compounds, of which GR127935 is the prototype (Clitherow et al., 1994). Recently, some specific antagonists have been described for h5-HT$_{1D}$R (BRL-15572, Price et al., 1997) and h5-HT$_{1B}$R [SB216641 (Price et al., 1997) and SB224289 (Gaster et al., 1998)] [see Halazy et al. (1997) for review].

Besides their distinct localization, differences in signal transduction properties could underlie a distinct function of 5-HT$_{1B}$R and 5-HT$_{1D}$R. Several studies established that 5-HT$_{1B}$R and 5-HT$_{1D}$R are negatively coupled to adenylate cyclase through pertussis toxin-sensitive G-proteins in vivo (Hoyer and Schoeffter, 1988) as well as in vitro (Hamblin and Metcalf, 1991; Weinshank et al., 1992). This implies the involvement of members of the G$\alpha_{i/o}$ family of G-proteins. However, to our knowledge, a detailed study of the specific G-protein subtypes that interact with the 5-HT$_{1D}$R or 5-HT$_{1B}$R has not been reported.

As proved by a growing amount of studies, the baculovirus expression system in insect cells has evolved to an established tool for the study of GPCRs (Butkerait et al., 1995; Wenzel-Seifert et al., 1998). This system offers the possibility to express high amounts of a well-defined receptor in a very low background of potentially interfering GPCR. The receptor can be obtained in a virtually noncoupled form (when highly expressed by itself) and in a highly coupled form (when coexpressed with high amounts of appropriate G-proteins). Thus, the expression of a well-defined combination of G-proteins can be superposed to that of the receptor. As such, the baculovirus-mediated GPCR expression in insect cells represents a unique medium for the study of structural changes induced by the interaction with a G-protein and provides experimental argumentation for the prevailing two-state model for activation of GPCRs. Finally, because it can be used for [^{35}S]GTPγS binding experiments and, more specifically for serotonin receptors, for serum-free growth conditions, the Sf9 insect cell expression system is very sensitive at unmasking constitutive activity of GPCRs (Hartman and Northup, 1996; Barr and Manning, 1997b; Wenzel-Seifert et al., 1998). Discerning this agonist-independent receptor activity is crucial because of its implications for therapeutic approaches, e.g., as in the need of inverse agonists for functional blockade of constitutively active receptors.

We aimed at studying h5-HT$_{1D}$R by reconstituting its interaction with G-proteins in a baculovirus-based expression system. Specificity of G-protein coupling was analyzed using [^{3}H]5-HT concentration binding experiments. Constitutive receptor activity was evidenced in [^{35}S]GTPγS binding assays using the inverse agonist ocaperidone. Further characterization of ocaperidone binding on h5-HT$_{1D}$R revealed the profound differences in binding properties of this ligand depending on G-protein coupling, hence providing further evidence for the occurrence of the receptor in different conformations.

Experimental Procedures

Materials. Sf-900 II serum-free medium was purchased from Life Technologies (Paisley, UK). The AcGP67 expression vector was from Pharmingen (San Diego, CA). Virions producing the rat Gα_{i1}, Gα_{i2}, Gα_{i3}, and Gα_{o} subunits were obtained from Dr. J. Garrisson (Department of Pharmacology, University of Virginia, Health Science Center, Charlottesville, VA), the mouse Gα_{q} was kindly provided by Dr. A. Gilman (Department of Pharmacology, University of Texas, Southwestern Medical Center, Dallas, TX), and the bovine G$\beta_1\gamma_2$ helpervector was a gift of Dr. T. Haga (Department of Biochemistry, Institute for Brain Research, Faculty of Medicine, Tokyo University, Hongo, Tokyo). The C6 glioma cell line expressing h5-HT$_{1D}$R was obtained from BioMed Consulting (Madrid, Spain). The antisera for h5-HT$_{1D}$R, h5-HT$_{1B}$R, G$\alpha_{i/o}$, Gβ_1, and Gγ_2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and that for Gα_{q} from Chemicon International (Temecula, CA). The peroxidase-conjugated anti-rabbit secondary antibody was obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PE). Chemiluminescent Western detection kit (ECL) was from Amersham Pharmacia Biotech (Little Chalfont, UK). Molecular weight markers were from Bio-Rad (Hercules, CA). [^{3}H]5-HT (80 to 130 Ci/mmol) and [^{35}S]GTPγS (>1000 Ci/mmol) were obtained from Amersham Pharmacia Biotech. [^{3}H]Ocaperidone (18.3 Ci/mmol) was labeled at the Janssen Research Foundation. Alniditan, ocaperidone, and ketanserin are original products from Janssen Pharmaceutica. 5-HT and dihydroergotamine-mesylate were from Acros Oganics (Geel, Belgium), methiothepin was purchased from Hoffmann-La Roche, GR127935 and pargyline were obtained from Sigma-Aldrich (St. Louis, MO). Sumatriptan was kindly donated by Glaxo. GTPγS, GDP, and Gpp(NH)p were from Roche Molecular Biochemicals (Mannheim, Germany). Other chemicals were from Sigma-Aldrich or Merck (Belgium). Stock solutions of compounds were prepared in dimethyl sulfoxide or in assay buffer. Bradford reagent for protein concentration determination was from Bio-Rad. The Ultra Turrax homogenizer was from Janke and Kunkel (Staufen, Germany), the Brandel 96-sample harvester from Brandel (Montreal, Canada). GF/B filters were from Whatman (Kent, UK). The liquid scintillation spectrometer (TriCarb) and the scintillation fluid (Ultima Gold MV) were from Packard (Meriden, CT). The Prism program was purchased from GraphPad Software (San Diego, CA).

Receptor Expression. The reading frame of h5-HT$_{1D}$R and h5-HT$_{1B}$R cDNAs were joined to the Baculo Ac secretion signal in the AcGP67 vector to improve expression. In this vector, transcription of the cDNA of interest is driven by the polyhedrin promoter. The G-protein cDNAs were expressed from the pVL1393 vector (Pharmingen, San Diego, CA). Sf9 cells were cultured, as described previously (Butkerait et al., 1995), in spinner flasks using serum-free Sf-900 II medium. Exponentially growing Sf9 cells were infected with a multiplicity of infection of 2 for each virus added. Cells were harvested at 48 h postinfection.

Membrane Preparation. For crude membrane preparation, cells were harvested (2,000*g*, 10 min) and the pellet resuspended in phosphate-buffered saline with subsequent centrifugation (20,000*g*, 10 min). The procedure was repeated twice. The washed cell pellet was frozen or immediately processed as follows. Cells were resuspended in hypotonic 10 mM Tris-HCl (pH 7.4) buffer, homogenized for 20 s (Ultra-Turrax homogenizer) and the homogenate centrifuged (30,000*g*, 30 min). The supernatant was discarded, and membranes were resuspended in 50 mM Tris-Cl (pH 7.4) buffer and stored in aliquots at −70°C at a concentration of ~1 mg/ml protein. Finally, after thawing, the protein concentration was determined using the Bradford assay with BSA as standard protein. The membranes were used in ligand concentration-binding, competition binding, [^{35}S]GTPγS binding and Western blotting experiments.

Radioligand Binding Assays. Membrane preparations were thawed on ice and diluted in 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$, 1 mM EGTA, and 10 μM pargyline (monoamine oxidase inhibitor). Incubation mixtures of 0.5 ml were composed of 0.4 ml of crude membrane preparation containing approximately 10 μg of membrane protein, 50 μl of radioligand, and 50 μl of solvent or competitor or alniditan (10 μM final concentration, for measuring nonspecific binding of the radioligand). Incubation was performed in the dark for 60 min at 25°C and stopped by rapid

filtration under suction over GF/B filters (1.5-cm diameter) using a Brandel 96 sample harvester. Filters were rinsed two times with 3 ml of 50 mM Tris-HCl (pH 7.4) buffer cooled in ice-water. Radioligand concentration-binding experiments were performed with [^{3}H]5-HT (specific activity, 80 to 130 Ci/mmol) using 10 to 12 concentrations ranging from 0.125 to 20 nM or with [^{3}H]ocaperidone (specific activity, 18.3 Ci/mmol), using 8 to 10 concentrations ranging from 0.125 to 4 nM. The same buffer and incubation conditions were used for [^{3}H]5-HT and for [^{3}H]ocaperidone concentration binding experiments. The effect of 100 μM Gpp(NH)p on binding of both [^{3}H]5-HT and [^{3}H]ocaperidone and of various concentrations of ocaperidone on the binding of [^{3}H]5-HT were investigated. In competition binding experiments, the effect of 12 concentrations of the cold competitor (ranging from 10^{-4} to 10^{-11} M for agonists and as low as 10^{-14} M for antagonists) was investigated on the binding of [^{3}H]5-HT (3 nM) or [^{3}H]ocaperidone (1 nM).

Radioligand concentration binding isotherms were calculated using computerized nonlinear regression analysis of a rectangular hyperbola (GraphPad software). The maximal number of binding sites (B_{max}) and apparent equilibrium dissociation constant (K_d) values for the radioligand were derived from the calculated curve. Competition binding curves were analyzed by nonlinear regression analysis of a sigmoid curve. IC_{50} values (concentration of competitor that inhibits 50% of specific radioligand binding) were derived from the calculated curves. Apparent K_i values were calculated as $K_i = [IC_{50}]/[1 + c/K_d]$ (where c is the radioligand concentration and K_d is the apparent equilibrium dissociation constant of the radioligand).

[^{35}S]GTPγS Binding Assays. Membrane samples were thawed on ice and suspended at a concentration of approximately 15 μg of protein/ml in 50 mM Tris-HCl buffer (pH 7.4), 0.5 mM $MgCl_2$, 50 mM NaCl, 1 μM GDP, and 10 μM pargyline. Incubation mixtures of 1 ml were preincubated with the test compounds for 5 min at 30°C (compounds were added at six concentrations) before addition of 10 μl of 10 nM [^{35}S]GTPγS (>1000 Ci/mmol). The incubation was run for 30 min at 30°C and stopped by rapid filtration over GF/B filters as described above. Filter-bound radioactivity was counted in a liquid scintillation spectrometer after overnight incubation of the filters in 3 ml of scintillation fluid. Basal [^{35}S]GTPγS binding was counted in the absence of compounds. Stimulation of [^{35}S]GTPγS binding by agonists was presented as a percentage over basal and was calculated as 100 × the difference between stimulated and basal binding (in cpm) divided by the amount of basal binding (in cpm). 5-HT or ocaperidone concentration-response curves for increases/decreases in [^{35}S]GTPγS binding were analyzed by nonlinear regression using the Prism software (GraphPad). EC_{50} values (concentration of compound at which 50% of its own maximal effect is obtained) were derived from the calculated curves.

Western Blotting. Membrane proteins (10 μg for the analysis of h5-HT_{1D}R, h5-HT_{1B}R, Gα and Gβ subunits or 5 μg for analysis of Gγ) were treated at 37°C for 2 h in lysis buffer (62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 5% SDS, 0.01% bromphenol blue, and 1% β-mercaptoethanol) and separated by SDS-polyacrylamide gel electrophoresis using standard techniques. Proteins were transferred to nitrocellulose membranes. Immunodetection was performed with a 1000-fold dilution of the antisera for h5-HT_{1B}R, $G\alpha_{i/o\ common}$, $G\alpha_q$, $G\beta_1$, and $G\gamma_2$. The "$G\alpha_{i/o\ common}$" antibody is cross-reactive for all members of the $G\alpha_{i/o}$ family. The antiserum for h5-HT_{1D}R was used at a concentration of 3 μg/ml. The peroxidase-coupled anti-rabbit secondary antibody was used in a final dilution of 1:5000. Enhanced chemiluminescence was used to visualize the bands as prescribed by the commercial supplier. Detection of each protein was performed using separate gels. Different exposure times were applied.

Results

Immunodetection of h5-HT_{1D}R, h5-HT_{1B}R, Gα-Proteins, and G$\beta_1\gamma_2$ Expressed in Sf9 Cells.

Recombinant baculoviruses containing the cDNAs encoding the human h5-HT_{1D}R and h5-HT_{1B}R (in fusion with the signal peptide of the baculovirus GP67 protein), diverse Gα subunits ($G\alpha_{i1}$, α_{i2}, α_{i3}, α_o, and α_q) and the $G\beta_1\gamma_2$ subunits were used to perform coexpressions in Sf9 cells. H5-HT_{1D}R was expressed either alone, together with a mixture of the $G\alpha_{i1}$, α_{i2}, α_{i3}, and α_o proteins (indicated as $G\alpha_{i/o}$ on the figures) or with each of these Gα subunits individually. Each of these coinfections was performed with and without $G\beta_1\gamma_2$. As a control, h5-HT_{1D}R was coexpressed with $G\alpha_q\beta_1\gamma_2$. For comparison, h5-HT_{1B}R was also introduced in Sf9 cells, either alone or together with $G\alpha_{i1}\beta_1\gamma_2$. Expression of the different proteins was assessed in Western blotting experiments performed on crude membrane preparations issued from the diverse coinfection materials (Fig. 1). For h5-HT_{1D}R, multiple closely spaced bands around the expected molecular weight (42 kDa) were detected. Multiple immunoreactive bands were also detected for the 5-HT_{1A}, β1-adrenergic, and *N*-formyl peptide receptors when expressed in Sf9 cells, and these were postulated to represent biosynthetic receptor precursors or improperly processed receptors (Quehenberger et al., 1992; Butkerait et al., 1995). No immunoreactive band was detected with the same antibody for membrane samples from uninfected Sf9 cells or cells expressing h5-HT_{1B}R (not shown), which confirms the specificity of the signal. Using an antiserum for h5-HT_{1B}R, a predominant band could be detected in membrane preparations from Sf9 cells expressing h5-HT_{1B}R (±40 kDa). This difference (i.e., one predominant band for h5-HT_{1B}R versus multiple immunoreactive bands for h5-HT_{1D}R) suggests that these two receptors are processed in a different way or to a different extent in Sf9 cells. No signal was seen for membrane preparations from cells expressing h5-HT_{1D}R using this anti-h5-HT_{1B}R antibody (Fig. 1). The diverse $G\alpha_{i/o}$ proteins (±40

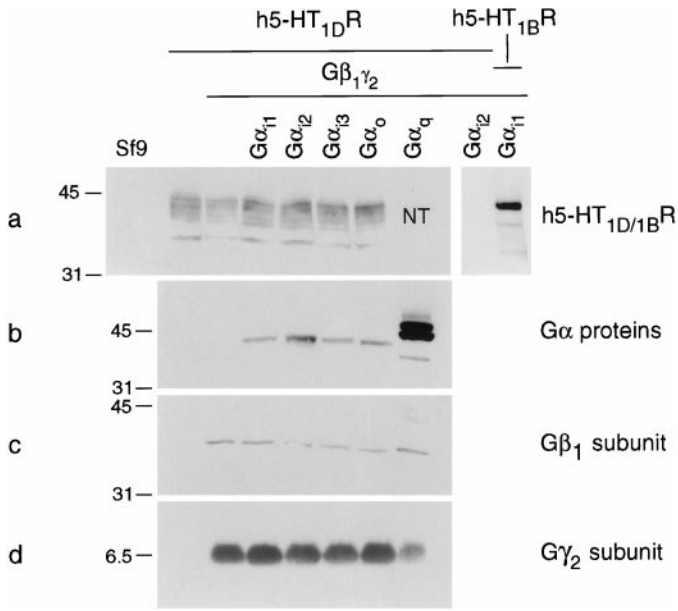


Fig. 1. Immunoblot analysis of $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, $G\alpha_q$, $G\beta_1$, $G\gamma_2$, and h5-HT_{1D}R and h5-HT_{1B}R expressed in Sf9 cells. The analysis was performed on membranes of uninfected Sf9 cells (Sf9) or Sf9 cells expressing h5-HT_{1D}R alone, together with $G\beta_1\gamma_2$ or together with $G\beta_1\gamma_2$ and each Gα subtype individually, as indicated at the top. H5-HT_{1B}R was detected in membranes from cells coexpressing this receptor with $G\alpha_{i1}\beta_1\gamma_2$. Antisera against h5-$HT_{1D}$R (a, left panel) or h5-$HT_{1B}$R (a, right panel), $G\alpha_{i/o}$ or $G\alpha_q$ (b), $G\beta_1$ (c), and $G\gamma_2$ (d) were used to visualize the proteins expressed. NT: not tested.

kDa) could be detected using an antibody ($G\alpha_{i\ common}$) cross-reactive for all members of this family of G-proteins. Some variations were apparent in the expression levels of the different $G\alpha$ subunits. For $G\alpha_q$, two immunoreactive bands were detected, as described previously (Hepler et al., 1993). The $G\beta_1$ (36 kDa) and $G\gamma_2$ (6.5 kDa) proteins showed comparable expression levels from one infection to the other. With the antibodies applied, no endogenous cross-reactive G-protein homologues of the mammalian $G\beta_1$, $G\gamma_2$, or $G\alpha_{i/o}$ proteins were visualized in the membrane samples from uninfected Sf9 cells, despite the broad detection range of the "$G\alpha_{i\ common}$" antibody. The absence of endogenous cross-reactive G-proteins was also reported by others [Grünewald et al. (1996); Leopoldt et al. (1997) and Wenzel-Seifert et al. (1998)].

Analysis of the Effect of G-Protein Coexpression on the [^{3}H]5-HT Binding Properties of h5-HT$_{1D}$R Produced in Sf9 Cells. Membrane material from cells expressing diverse combinations of receptor and G-proteins were used for [^{3}H]5-HT concentration binding experiments to estimate receptor expression levels and agonist affinity. A representative example of the [^{3}H]5-HT concentration binding curves obtained with membrane material from Sf9 cells expressing h5-HT$_{1D}$R alone or in combination with $G\alpha_{i1}\beta_1\gamma_2$ or $G\alpha_q\beta_1\gamma_2$ is shown in Fig. 2. The mean apparent K_d values derived from the computerized curve fitting of the [^{3}H]5-HT binding data for each combination of receptor and G-protein are summarized in Table 1. Oscillations in the B_{max} values appeared independent from the combination of proteins expressed. The range of B_{max} values obtained are indicated in Table 1. Because the Sf9 expression system does not allow expression ratios between receptor and G-proteins to remain strictly constant from one infection to the other, experiments were performed on material issued from two to five independent coinfections to evaluate possible effects on apparent K_d values. For [^{3}H]5-HT, an apparent mean K_d of 7 nM was measured for h5-HT$_{1D}$R when expressed alone. On average, addition of a single $G\alpha_{i/o}$ subunit or a set of four $G\alpha_{i/o}$ proteins led to an almost 2-fold increase in receptor affinity for [^{3}H]5-HT. Reconstitution of the G-protein trimer by further addition of the $G\beta_1\gamma_2$ subunits switched the receptor population to a high affinity state, increasing the agonist affinity of the receptor almost 5-fold as compared with the receptor expressed alone. For certain membrane preparations from cells overexpressing the receptor without G-protein, a minor high [^{3}H]5-HT affinity component was detected, which probably represents a fraction of h5-HT$_{1D}$R coupled to endogenous G-proteins. When detected, this component represented maximally 15% of the total receptor population. The coexpression of $G\beta_1\gamma_2$ or $G\alpha_q\beta_1\gamma_2$ with the receptor had no significant effect on agonist affinity of the receptor. For comparison, the [^{3}H]5-HT affinity of h5-HT$_{1B}$R expressed in the same expression setup was determined. The relatively low agonist affinity of h5-HT$_{1B}$R when expressed alone (22 nM) was increased about 15-fold by the addition of $G\alpha_{i1}\beta_1\gamma_2$ (1.5 nM).

An index for the functionality of the reconstituted receptor-G-protein coupling is its GTP sensitivity. [^{3}H]5-HT concentration binding experiments were performed in the presence of 100 μM Gpp(NH)p on membrane material from cells expressing h5-HT$_{1D}$R alone or with $G\alpha_q\beta_1\gamma_2$, $G\alpha_{i1}\beta_1\gamma_2$, $G\alpha_{i/o}$, or

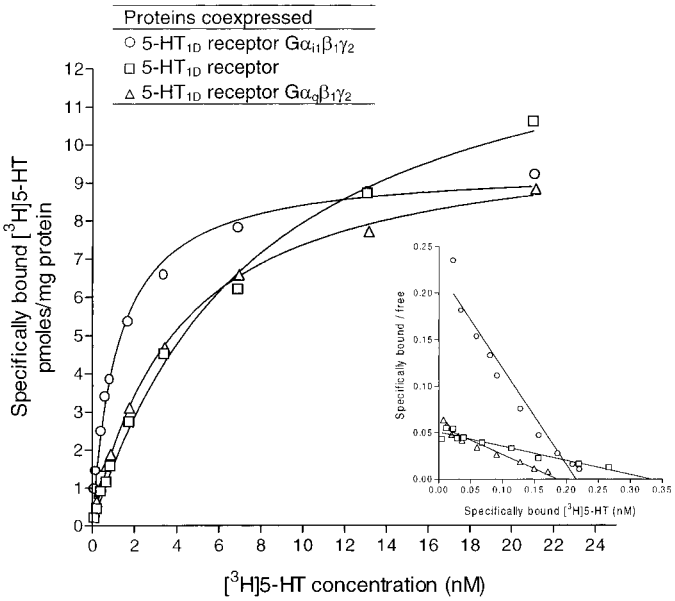


Fig. 2. Effect of G-proteins on [^{3}H]5-HT binding at 25°C to h5-HT$_{1D}$R coexpressed in Sf9 cells. Concentration binding isotherms and Scatchard plots (inset) for [^{3}H]5-HT binding on membranes of Sf9 cells expressing h5-HT$_{1D}$R alone, together with $G\alpha_{i1}\beta_1\gamma_2$ or $G\alpha_q\beta_1\gamma_2$, are shown. The figure represents data obtained in one of several experiments. K_d and B_{max} values were derived for each individual experiment using nonlinear curve fitting to the rectangular hyperbola, and mean values are summarized in Table 1.

TABLE 1

Effect of G-protein coexpression and Gpp(NH)p addition on [^{3}H]5-HT binding to 5-HT$_{1D}$R and 5-HT$_{1B}$R expressed in Sf9 cells

[^{3}H]5-HT concentration binding experiments (0.125 to 20 nM, 10 to 12 points) were performed as described in the text on membrane material from Sf9 cells coexpressing h5-HT$_{1D}$R or h5-HT$_{1B}$R with the indicated G-protein combinations. One-site binding analysis was performed on the data. Mean apparent equilibrium dissociation constant (K_d) and range of maximal number of binding sites (B_{max}) for [^{3}H]5-HT binding to h5-HT$_{1D}$R and h5-HT$_{1B}$R are shown. (Examples of curves are shown in Fig. 1.) $G\alpha_{i/o}$ indicates that the receptor was coexpressed with $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_o$ subunits simultaneously. Experiments were performed with cell membranes from two to five separate infections per case. Because infection efficiency and protein expression can vary with separate infections, the lowest and highest B_{max} value is given instead of a mean value.

Coexpressed Proteins	Gpp(NH)p addition	K_d (Mean ± S.D.)	B_{max} Range	n
		nM	*pmol/mg protein*	
5-HT$_{1D}$R	No	6.7 ± 1.6	8.4–18.9	12
	Yes	9.5 ± 1.2	10.9–19.5	3
5-HT$_{1D}$R $G\alpha_{i1}$	No	4.3 ± 1.2*	1.7–13.0	6
5-HT$_{1D}$R $G\alpha_{i2}$	No	3.1 ± 0.6**	8.2–17.1	5
5-HT$_{1D}$R $G\alpha_{i3}$	No	4.0 ± 1.7*	7.9–22.17	5
5-HT$_{1D}$R $G\alpha_o$	No	3.7 ± 1.6*	3.8–14.7	6
5-HT$_{1D}$R $G\alpha_{i/o}$	No	3.7 ± 0.5*	14.1–17.2	3
	Yes	4.2 ± 1.1	12.6–17.2	3
5-HT$_{1D}$R $G\alpha_{i1}\beta_i\gamma_2$	No	1.6 ± 0.6***††	5.0–19.5	12
	Yes	5.4 ± 1.7^b	16.7–25.3	3
5-HT$_{1D}$R $G\alpha_{i2}\beta_1\gamma_2$	No	1.3 ± 0.3***††	3.1–15.6	8
5-HT$_{1D}$R $G\alpha_{i3}\beta_1\gamma_2$	No	1.6 ± 0.7***†	2.7–20.6	8
5-HT$_{1D}$R $G\alpha_o\beta_1\gamma_2$	No	1.7 ± 0.5***	3.7–20.1	8
5-HT$_{1D}$R $G\alpha_{i/o}\beta_1\gamma_2$	No	1.9 ± 0.2**††	12.2–17.6	3
	Yes	4.4 ± 1^a	13.0–17.2	3
5-HT$_{1D}$R $G\alpha_q\beta_1\gamma_2$	No	5.6 ± 1.8	6.5–24.2	12
	Yes	7.9 ± 1.5	14.5–26.6	3
5-HT$_{1D}$R $G\beta_1\gamma_2$	No	5.3 ± 2.5	7.1–10.5	4
5-HT$_{1B}$R	No	21.87 ± 1.50	3.8–10	3
5-HT$_{1B}$R $G\alpha_{i1}\beta_1\gamma_2$	No	1.49 ± 0.20***	3.94–9.9	3
	Yes	13.71 ± 3.86^b	3.28–8.74	3

Statistical analysis was performed using Student's t test: The asterisks (*$P <$ 0.05, **$P < 0.01$, and ***$P < 0.001$) refer to the differences in K_d values with the receptor expressed alone, and the dagger symbols ($^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$) refer to the difference in K_d values with the receptor expressed with the corresponding $G\alpha$ subunit without $G\beta_1\gamma_2$. ^{a} ($P < 0.05$) and ^{b} ($P < 0.01$) refer to the differences in K_d values with the receptor without Gpp(NH)p addition.

$G\alpha_{i/o}\beta_1\gamma_2$. Addition of Gpp(NH)p had no significant effect on [^{3}H]5-HT binding for the receptor expressed alone or in conjunction with $G\alpha_q\beta_1\gamma_2$ or $G\alpha_{i/o}$, whereas the [^{3}H]5-HT affinity of the receptor coexpressed with $G\alpha_{i1}\beta_1\gamma_2$ and $G\alpha_{i/o}\beta_1\gamma_2$ was significantly reduced (Table 1). B_{max} levels remained unaffected by the Gpp(NH)p treatment (see also Table 3). Similarly, in the case of h5-HT_{1B}R, the agonist affinity of the $G\alpha_{i1}\beta_1\gamma_2$-coexpressed receptor was markedly reduced by Gpp(NH)p treatment.

Pharmacological Profile of h5-HT_{1D}R. To test the pharmacological robustness of our expression setup for h5-HT_{1D}R, the capacity of diverse compounds for displacing 3 nM [^{3}H]5-HT was assessed in competition binding experiments. Membrane material of cells expressing the receptor alone, together with $G\alpha_{i1}\beta_1\gamma_2$, $G\alpha_o\beta_1\gamma_2$, or $G\alpha_q\beta_1\gamma_2$ were used. Agonists, a partial agonist and antagonists for h5-HT_{1D}R [as shown in signal transduction assays (Pauwels, 1997; Lesage et al., 1998)], were selected from diverse major chemical structural classes and tested. Tested compounds, K_i and pK_i values derived from the inhibition curves are listed in Table 2. Comparing pK_i values obtained for h5-HT_{1D}R expressed in Sf9 and in a stably transformed C6 glioma cell line (Leysen et al., 1996), the order of potency of the compounds appeared to be maintained. The pK_i values obtained for agonists using membrane material containing the receptor coexpressed with $G\alpha_{i1}\beta_1\gamma_2$ are similar to the values obtained with C6 glioma cell membranes. For the agonists, the pK_i values obtained with cell membranes containing the receptor alone and the receptor with $G\alpha_q\beta_1\gamma_2$ were slightly lower than those obtained with the receptor expressed in combination with $G\alpha_{i1}\beta_1\gamma_2$ or $G\alpha_o\beta_1\gamma_2$. For the three antagonists, pK_i values obtained for the receptor in noncoupled state (expressed alone or in the presence of $G\alpha_q\beta_1\gamma_2$) were up to two orders of magnitude higher than for the receptor in coupled state (expressed in the presence of $G\alpha_{i1}\beta_1\gamma_2$ or $G\alpha_o\beta_1\gamma_2$). Inhibition curves showing the competition by ocaperidone of [^{3}H]5-HT binding on h5-HT_{1D}R coexpressed with various G-proteins (in the presence or absence of Gpp(NH)p) are represented in Fig. 3. pIC_{50} values and the Hill coefficient associated with these curves, when fitting to a one-site model with variable slope, are also shown in Fig. 3. Displacement of [^{3}H]5-HT from the noncoupled h5-HT_{1D}R did not fit to a one-site competition curve with a Hill coefficient of 1. The fit could be improved by using a one-site model with a shallow slope (pIC_{50} = 9.1; Hill coefficient = 0.4) or a two-site model, with 42% of the receptors having a high ocaperidone affinity (pIC_{50} = 10.1) and the remainder a low affinity (pIC_{50} = 7.1). The two-site model would imply the existence of different binding sites or a nonhomogenous receptor population, the one-site model would imply a mode of inhibition inconsistent with competitive inhibition. Treatment of membranes containing the $G\alpha_{i1}\beta_1\gamma_2$- or $G\alpha_o\beta_1\gamma_2$-coexpressed receptor with Gpp(NH)p caused a decrease of the Hill coefficient of the inhibition curve for ocaperidone from unity to 0.44 as well as an increase of the potency of ocaperidone to displace [^{3}H]5-HT.

Analysis of [^{3}H]Ocaperidone Binding on h5-HT_{1D}R. As for [^{3}H]5-HT (see above and Table 1), the effect of G-protein and Gpp(NH)p addition on [^{3}H]ocaperidone binding on h5-HT_{1D}R was assessed in concentration binding experiments. Representative examples of the [^{3}H]ocaperidone concentration binding curves obtained with membrane material from Sf9 cells expressing h5-HT_{1D}R alone, in conjunction with $G\alpha_{i1}\beta_1\gamma_2$ (with or without Gpp(NH)p) or $G\alpha_q\beta_1\gamma_2$ are shown in Fig. 4. The mean apparent K_d and B_{max} values derived from the computerized curve fitting of the [^{3}H]ocaperidone binding data for each combination of receptor and G-protein are summarized in Table 3. The B_{max} values obtained with [^{3}H]5-HT concentration binding experiments on the same membrane preparation are shown for comparison. Subnanomolar K_d values were obtained for the three membrane preparations tested, and no effect of Gpp(NH)p addition on the apparent binding affinity of [^{3}H]ocaperidone was seen. For noncoupled receptor material, the B_{max} values obtained with [^{3}H]ocaperidone were slightly higher as than those detected with [^{3}H]5-HT. This might be due to the existence of an h5-HT_{1D}R component with low [^{3}H]5-HT affinity, which is not covered by the [^{3}H]5-HT concentration range used. For membranes from cells coexpressing h5-HT_{1D}R and $G\alpha_{i1}\beta_1\gamma_2$, B_{max} values detected with [^{3}H]ocaperidone were one fourth of those detected with [^{3}H]5-HT. Gpp(NH)p treatment of membrane material containing the coupled receptor led to a 3-fold increase of (high affinity) [^{3}H]ocaperidone binding sites.

Effect of 5-HT and Ocaperidone on h5-HT_{1D}R Activity Assessed by Measurement of [^{35}S]GTPγS Binding to $G\alpha_{i1}$. The agonist and antagonist/inverse agonist activity of 5-HT and ocaperidone, respectively, were investigated in

TABLE 2

Inhibition of [^{3}H]5-HT binding to membranes of Sf9 cells coexpressing the 5-HT_{1D}R and diverse G-protein combinations by 5-HT receptor ligands

Radioligand binding was performed with 3 nM [^{3}H]5-HT as described in the text. pIC_{50} values were derived from individual curves and used to calculate pK_i values. K_i values (nM) were added between brackets to facilitate comparison with Table 1. Values for membrane material from a C6-glioma cell line stably expressing the 5-HT_{1D}R were added for comparison and are from Leysen et al. (1996).

Competitor	5-HT_{1D}R	5-HT_{1D}R $G\alpha_{i1}\beta_1\gamma_2$	5-HT_{1D}R $G\alpha_o\beta_1\gamma_2$	5-HT_{1D}R $G\alpha_q\beta_1\gamma_2$	C6 Glioma
Agonists					
5-HT	8.4 ± 0.1 (3.8)[a]	8.9 ± 0.2* (1.3)	8.9 ± 0.1* (1.2)	8.3 ± 0.1 (5.3)	8.5 (2.9)
Alniditan	8.4 ± 0.5 (3.9)	9.0 ± 0.3 (1.0)	8.8 ± 0.6 (1.7)	8.4 ± 0.8 (3.7)	9.4 (0.4)
Sumatriptan	7.9 ± 0.5 (12.8)	8.5 ± 0.2 (2.9)	8.3 ± 0.2 (4.6)	8.0 ± 0.3 (10.0)	8.5 (3.5)
Dihydroergotamine	8.9 ± 0.4 (1.1)	9.1 ± 0.4 (0.9)	9.1 ± 0.5 (0.9)	8.5 ± 0.2 (3.3)	9.2 (0.7)
Partial agonist					
GR127935	8.6 ± 0.5 (2.6)	8.5 ± 0.1 (3.2)	9.0 ± 0.7 (1.0)	8.4 ± 0.4 (4.0)	8.6 (2.6)
Antagonists					
Ocaperidone	10.4 ± 1.7 (0.04)	7.0 ± 0.2* (102)	7.0 ± 0.7* (101)	9.9 ± 0.7 (0.3)	8.8 (1.5)
Methiothepin	>14[b]	6.5 ± 0.1 (344)	6.8 ± 0.2 (170)	>14[b]	7.6 (28)
Ketanserin	7.2 ± 0.5 (66)	5.8 ± 0.6* (1550)	5.9 ± 0.3* (1370)	7 ± 0.6 (96)	7.5 (31)

Statistical analysis was performed using Student's t test: The asterisk (* $P < 0.05$) refers to the differences in K_i values with the noncoupled receptor.

[a] pK_i values (−log M) (mean ± S.D., n = 3) and K_i values (nM) (inside parens).

[b] >14 indicates that the lowest concentration of competitor tested (10^{-14} M) produced more than 50% inhibition of binding.

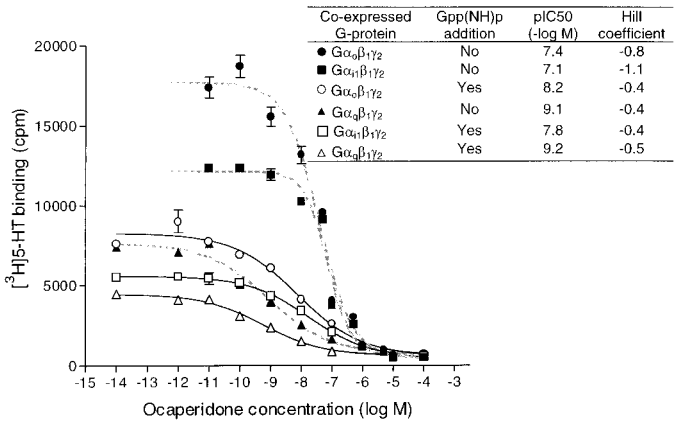


Fig. 3. Inhibition at 25°C of [^{3}H]5-HT binding by ocaperidone on h5-HT$_{1D}$R. Membrane material from Sf9 cells expressing h5-HT$_{1D}$R with $G\alpha_{i1}\beta_1\gamma_2$, $G\alpha_o\beta_1\gamma_2$, or $G\alpha_q\beta_1\gamma_2$ was labeled with 3 nM [^{3}H]5-HT in the presence of increasing amounts of ocaperidone with or without 100 μM Gpp(NH)p. A single experiment (with points determined in duplicate) is shown that is representative of a total of three independent experiments. Curves were generated by fitting to a sigmoidal curve with variable slope. pIC$_{50}$ values and Hill coefficients derived from this fitting are shown in the insert.

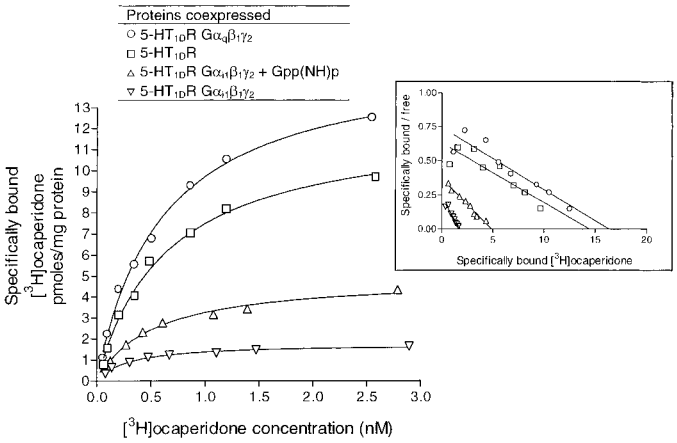


Fig. 4. Effect of G-proteins and Gpp(NH)p on [^{3}H]ocaperidone binding (25°C) to h5-HT$_{1D}$R expressed in Sf9 cells. Concentration binding isotherms and Scatchard plots (inset) for [^{3}H]ocaperidone binding to membranes of Sf9 cells expressing h5-HT$_{1D}$R alone, together with $G\alpha_{i1}\beta_1\gamma_2$ (with or without addition of 100 μM Gpp(NH)p) or $G\alpha_q\beta_1\gamma_2$, are shown. The figure represents data obtained in one of three experiments. K_d and B_{max} values were derived for each individual experiment using nonlinear curve fitting to the rectangular hyperbola, and mean values are summarized in Table 3.

[^{35}S]GTPγS binding experiments using membrane preparations of Sf9 cells coexpressing h5-HT$_{1D}$R and $G\alpha_{i1}\beta_1\gamma_2$. Concentration-effect curves with derived pEC$_{50}$ values are shown on Fig. 5. Typically, basal [^{35}S]GTPγS binding yielded about 4000 cpm. No or weak ($\leq$20%) enhancement of basal [^{35}S]GTPγS binding was obtained on incubation with 5-HT. Addition of ocaperidone, on the contrary, decreased [^{35}S]GTPγS binding levels by on average of 40%. The effect of ocaperidone could be competed by 5-HT, returning to the basal [^{35}S]GTPγS binding levels. Ocaperidone was found more potent at inhibiting the constitutive h5-HT$_{1D}$R-driven [^{35}S]GTPγS binding (pEC$_{50}$ = 7.8) than it was at displacing [^{3}H]5-HT from the coupled receptor (pK_i = 7). For 5-HT, the pEC$_{50}$ value for activation of [^{35}S]GTPγS binding (8.6) is close to the pK_d for the coupled receptor (8.8). Performing the [^{35}S]GTPγS binding experiments at higher salt concentrations (100 and 150 mM NaCl) did not significantly influence the basal [^{35}S]GTPγS binding levels and did not result in a decrease of the effect of ocaperidone on [^{35}S]GTPγS binding levels (data not shown). This indicates that the agonist-independent coupling of h5-HT$_{1D}$R can also be seen at physiological salt concentrations.

Analysis of the Type of Competition between 5-HT and Ocaperidone for Binding on h5-HT$_{1D}$R. The type of competition between 5-HT and ocaperidone binding was investigated by the analysis of [^{3}H]5-HT concentration binding curves in the presence and in the absence of different fixed concentrations of ocaperidone performed on membrane preparations from cells expressing h5-HT$_{1D}$R alone or together with $G\alpha_{i1}\beta_1\gamma_2$. Concentration binding curves and Scatchard plots are shown in Fig. 6.

In the case of the $G\alpha_{i1}\beta_1\gamma_2$-coexpressed receptor (Fig. 6, right panel), the affinity of [^{3}H]5-HT binding is affected by ocaperidone addition, without a decrease of B_{max} values. Scatchard transformation results in plots with decreasing slope on ocaperidone addition, whereas the intercept of the plots with the x-axis is maintained. This implies competitive inhibition of binding.

For the noncoupled receptor (Fig. 6, left panel), addition of ocaperidone at 1 and 10 nM led to a strong concentration-dependent decrease in apparent B_{max} values with a weak effect on the apparent K_d value. Performing a Scatchard transformation of these data (see Fig. 6, lower panel), a minor high agonist affinity component became apparent. This probably represents a pool of receptors coupled to en-

TABLE 3

Effect of G-protein coexpression and Gpp(NH)p addition on [^{3}H]ocaperidone binding to the 5-HT$_{1D}$R

[^{3}H]Ocaperidone concentration binding experiments (0.125 to 4 nM, 8 to 10 points) were performed as described in the text on membrane material from Sf9 cells expressing the h5-HT$_{1D}$R alone, together with $G\alpha_{i1}\beta_1\gamma_2$ or $G\alpha_q\beta_1\gamma_2$. Mean apparent equilibrium dissociation constant (K_d) and maximal number of binding sites (B_{max}) for [^{3}H]ocaperidone binding to the h5-HT$_{1D}$R are shown. (Examples of binding curves are shown in Fig. 4.) For comparison, the B_{max} values obtained with [^{3}H]5-HT for the same membrane preparation are shown.

G-Protein Coexpressed	Gpp(NH)p Added	[^{3}H]Ocaperidone		[^{3}H]5-HT	
		K_d (Mean ± S.D.)	B_{max} (Mean ± S.D.)	B_{max} (Mean ± S.D.)	n
		nM	*pmol/mg*	*pmol/mg*	
None	No	0.56 ± 0.17	31.6 ± 1.8	21.8 ± 6.1	3
None	Yes	0.56 ± 0.14	31.4 ± 0.7	22.5 ± 3.4	3
$G\alpha_{i1}\beta_1\gamma_2$	No	0.51 ± 0.33	5.9 ± 1.3	22.6 ± 2.4	3
$G\alpha_{i1}\beta_1\gamma_2$	Yes	0.47 ± 0.04	14.2 ± 2.3*	27.0 ± 4.6	3
$G\alpha_q\beta_1\gamma_2$	No	0.68 ± 0.07	40.8 ± 2.9	19.2 ± 5.2	3
$G\alpha_q\beta_1\gamma_2$	Yes	0.72 ± 0.40	34.8 ± 3.9	19.8 ± 2.7	2

Statistical analysis was performed using Student's t test: The asterisk (* $P < 0.05$) refers to the differences in B_{max} values with or without Gpp(NH)p addition for the receptor coexpressed with $G\alpha_{i1}\beta_1\gamma_2$.

dogenous G-proteins that is not accessible for ocaperidone. Complete receptor saturation could not be obtained because high [^{3}H]5-HT concentrations led to high background binding. Still, the shift of the plots on ocaperidone addition appears almost parallel. The plots are likely to have a different intercept with the *x*-axis, which would reflect a decrease in B_{max} with poor effect on the apparent K_d value of [^{3}H]5-HT.

Discussion

We took advantage of the baculovirus expression system in Sf9 cells to study intrinsic properties of h5-HT$_{1D}$R. Study of the equilibrium binding of the natural agonist 5-HT and of the inverse agonist ocaperidone on this receptor in coupled or noncoupled state as well as the study of h5-HT$_{1D}$R-driven activation of [^{35}S]GTPγS binding to Gα_{i1} allowed us to draw conclusions with regard to specificity of coupling, constitutive activity, and conformational changes of this receptor induced by G-protein interaction or ligand binding.

Reconstitution of h5-HT$_{1D}$R-G-Protein Coupling. [^{3}H]5-HT concentration binding experiments on membranes from Sf9 cells expressing h5-HT$_{1D}$R alone (Table 1) revealed an apparent K_d value in the range of the low affinity form of the receptor expressed in Chinese hamster ovary cells (Hamblin and Metcalf, 1991). In general, no high agonist-affinity receptor population was detectable in these experiments. However, Scatchard analysis of [^{3}H]5-HT concentration binding performed in the presence of 10 nM of the inverse agonist ocaperidone (Fig. 6) resulted in a curved plot. This suggests that, when most of the [^{3}H]5-HT low affinity component of h5-HT$_{1D}$R is (preferentially) masked by the inverse agonist ocaperidone, a small high affinity component for [^{3}H]5-HT binding becomes apparent. This might represent the receptor population coupled to endogenous G-proteins. These agonist high affinity sites are not accessible to ocaperidone at a concentration of 10 nM. When detected, these agonist high affinity sites did not exceed 15% of the total receptor population.

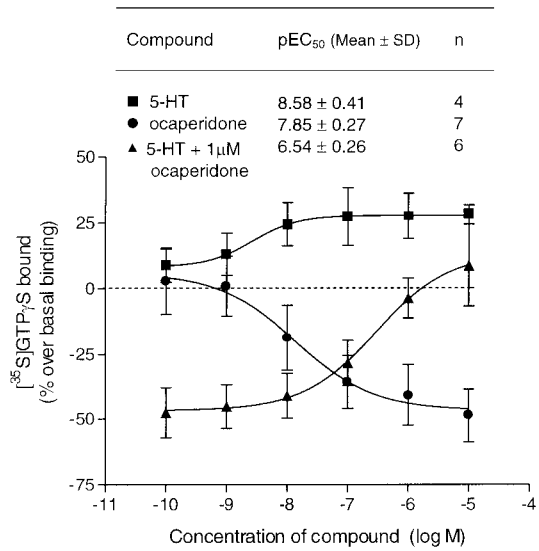


Fig. 5. Effect of 5-HT and ocaperidone on [^{35}S]GTPγS binding (30°C) to the Gα subunit of G$\alpha_{i1}\beta_1\gamma_2$ coexpressed with h5-HT$_{1D}$R. Membrane material of Sf9 cells coexpressing h5-HT$_{1D}$R and G$\alpha_{i1}\beta_1\gamma_2$ was preincubated with increasing amounts of 5-HT or ocaperidone before the addition of 0.1 nM [^{35}S]GTPγS. Basal [^{35}S]GTPγS binding was measured in the absence of compound. Competition between 5-HT and ocaperidone was tested by adding [^{35}S]GTPγS and 5-HT to membranes preparations preincubated with 1 μM ocaperidone. Results are expressed as a percentage of basal [^{35}S]GTPγS binding. Depicted data are mean ± S.D. of four to seven experiments. The mean of the individual pEC_{50} or pIC_{50} values ± S.D. as derived from the curve fitting are shown.

Coexpression of a Gα subunit of the G$_{i/o}$ family led to an increase in agonist affinity of the receptor (Table 1). An interaction of the Gα subunit alone with the receptor was already demonstrated for the 5-HT$_{1A}$R (with Gα_i) and rhodopsin (with Gα_t) (Kelleher and Johnson, 1988; Butkerait et al., 1995). Further addition of the G$\beta_1\gamma_2$ subunits, a $\beta\gamma$ combination capable of interacting with most Gα subunit types (Iñiguez-Lluhi et al., 1992), conferred a coupled phenotype to the main part of the receptor population (Table 1). The apparent K_d value of 1.5 nM [^{3}H]5-HT obtained for h5-HT$_{1D}$R in its high affinity form lies in the range of the K_d values (2.9 and 1.5 nM) determined in membrane material from C6 glioma or Chinese hamster ovary cell lines, respectively, stably expressing this receptor (Hamblin and Metcalf, 1991; Leysen et al., 1996). All Gα subunits of the G$_{i/o}$ family tested were able to induce a high affinity state of the receptor. This is in agreement with the pertussis toxin sensitivity of the signal transduction through the 5-HT$_{1D}$R in mammalian cells, which suggests that G$_{i/o}$ types of G-proteins are involved (Hamblin and Metcalf, 1991; Zgombick et al., 1993). As expected, G$_q$ did not enhance the agonist affinity of h5-HT$_{1D}$R, confirming the specificity of the interactions monitored. Lack of preference for a member of the G$_{i/o}$ protein family was also observed for the Sf9-expressed 5-HT$_{1A}$R (Butkerait et al., 1995). This suggests that the structural determinants of these receptors are not able to confer a high degree of specificity toward any member of the G$_{i/o}$ family. Under physiological circumstances, supplementary mechanisms might regulate the specificity of coupling such as, e.g., colocalization in tissues or microdomains.

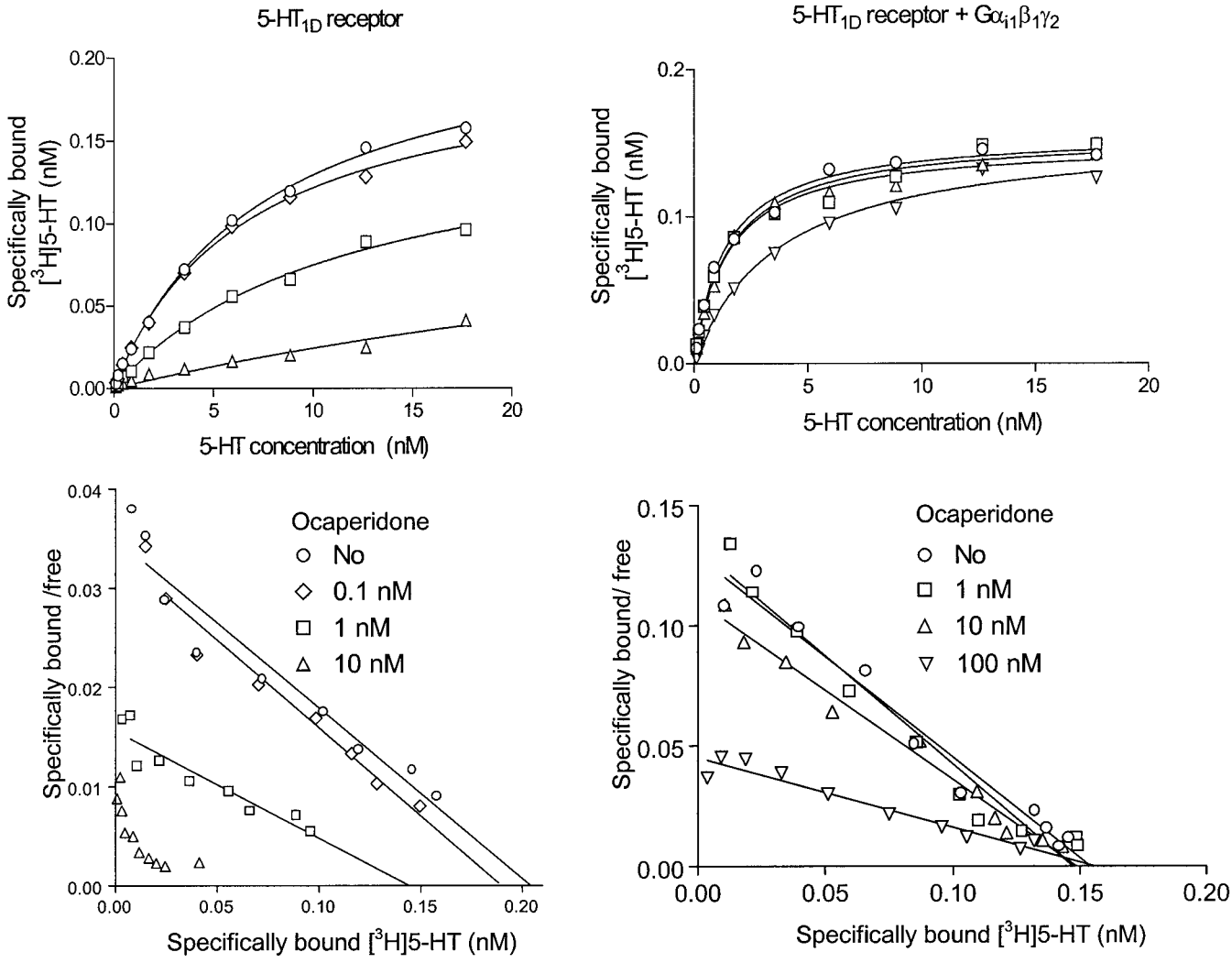
Our results are in contrast with a study of Clawges and coworkers (1997), which describes a weak coupling between the Sf9-expressed h5-HT$_{1D}$R and purified G$\alpha_{i/o}\beta\gamma$. Although the latter method permits a better quantitative control on the receptor/G-protein ratio applied, questions remain about the integrity and membrane insertion of G-proteins undergoing purification and solubilization procedures. Furthermore, In the coexpression approach, protein targeting occurs in a biosynthetic context.

The ability to be converted to a high affinity form by interaction with a G-protein is an index for functionality of h5-HT$_{1D}$R expressed in Sf9 cells. To prove the specificity of the receptor-G-protein interaction, its Gpp(NH)p sensitivity was assessed. A clear loss in agonist affinity of h5-HT$_{1D}$R coexpressed with G$\alpha_{i1}\beta_1\gamma_2$ was seen on addition of the nucleotide (see Table 1). The fact that the affinity for the coupled receptor cannot be lowered by Gpp(NH)p treatment to the value of the receptor expressed alone suggests that the isomerization of the receptor toward a high affinity state cannot be reversed totally by this treatment. It was suggested by Grünewald et al. (1996) that for some receptors NaCl must be present to fully convert high affinity sites into low affinity sites. Interestingly, for h5-HT$_{1B}$R, Gpp(NH)p caused a more dramatic loss in affinity (see Table 1), suggesting that these two receptors possess different coupling properties.

Constitutive Activity of h5-HT$_{1D}$R. A representative set of h5-HT$_{1D/1B}$R agonists and antagonists revealed a sim-

ilar pharmacological profile for h5-HT$_{1D}$R expressed in Sf9 and mammalian cells (Table 2). Agonists showed the tendency to displace [^{3}H]5-HT more efficiently at the coupled receptor, whereas all three antagonists clearly competed with [^{3}H]5-HT binding much more potently at the noncoupled receptor, indicative of an inverse agonistic character. Studies of h5-HT$_{1D}$R performed in mammalian cells already suggested this for methiothepin and ketanserin (Thomas et al., 1995; Pauwels, 1997). However, because serum, needed for mammalian cell growth, contains 5-HT levels that might trigger receptor activation, neutral antagonism is difficult to distinguish from inverse agonism in such a system. Furthermore, coupled and noncoupled receptors cannot unambiguously be distinguished in such a mammalian expression system.

G-protein activation, as measured in [^{35}S]GTPγS binding assays, made possible the monitoring of the efficacy of ligands and thus inverse agonism. We found that activation of h5-HT$_{1D}$R with an agonist mediated only a limited enhancement (25%) of the levels of [^{35}S]GTPγS bound by G$\alpha_{i1}\beta_1\gamma_2$



Ocaperidone concentration (nM)	5-HT$_{1D}$R K_d (nM)	5-HT$_{1D}$R B_{max} (pmoles/mg protein)
0	7.3	21.6
0.1	5.7	17.6
1	11.6	9.3
10	6.2	4.2
100	-	-

5-HT$_{1D}$R + G$\alpha_{i1}\beta_1\gamma_2$ K_d (nM)	B_{max} (pmoles/mg protein)
1.3	14.8
-	-
1.4	14.6
1.4	14.1
3.5	14.9

Fig. 6. Investigation of the competition between [^{3}H]5-HT and ocaperidone in the presence and in the absence of the G$\alpha_{i1}\beta_1\gamma_2$ heterotrimer. [^{3}H]5-HT concentration binding experiments (0.0125 to 18 nM, 10 points) were performed on membrane material from cells expressing h5-HT$_{1D}$R alone or in conjunction with G$\alpha_{i1}\beta_1\gamma_2$, in the presence of the indicated ocaperidone concentrations. The concentration binding isotherms (upper panels) and the Scatchard plots (lower panels) are shown. K_d and B_{max} values for the different curves are given. A single experiment representative of a total of three is represented.

(Fig. 5). The fact that $G\alpha_{i1}\beta_1\gamma_2$ has a relatively high degree of agonist-independent [^{35}S]GTPγS binding (Barr et al., 1997a) might have occluded activation of the receptor. Alternatively, activation of $G\alpha_{i1}\beta_1\gamma_2$ by an endogenous receptor may explain the high basal [^{35}S]GTPγS levels bound. However, as ocaperidone caused a 40% drop in [^{35}S]GTPγS binding, part of the basal [^{35}S]GTPγS binding was proved to be dependent on h5-HT_{1D}R. The loss in [^{35}S]GTPγS binding could be reverted by addition of 5-HT, indicating that ocaperidone and 5-HT act on the same receptor population. Our observations imply that ocaperidone shifts h5-HT_{1D}R to a conformation that is less accessible to the $G\alpha_i$-protein, lowering the amount of h5-HT_{1D}R-activated $G\alpha_i$-proteins that bind [^{35}S]GTPγS. The high level of agonist-independent [^{35}S]GTPγS binding driven by h5-HT_{1D}R was showed in this experiment. This constitutive activity is required for the detection of inverse agonism. The relevance of constitutive activity of wild type GPCRs has already been provided for, e.g., the 5-HT_{2C}, β-adrenergic, or muscarinic receptors (Hanf et al., 1993; Mewes et al., 1993; Barker et al., 1994). If the behavior of h5-HT_{1D}R expressed in our system indeed matches its behavior in physiological circumstances, inverse agonists might thus be needed to block h5-HT_{1D}R function for therapeutic purposes.

Analysis of the Binding of Ocaperidone to h5-HT_{1D}R. H5-HT_{1D}R conformations bound by ocaperidone were analyzed in a set of equilibrium binding experiments. Concentration binding experiments showed a saturation of the [^{3}H]ocaperidone binding sites at a concentration of about 3 nM. However, at this concentration, B_{max} values obtained with [^{3}H]ocaperidone equaled those seen with [^{3}H]5-HT only for the noncoupled receptor preparations. In the case of the coupled receptor, the number of [^{3}H]5-HT binding sites was 4-fold higher (Table 3). The latter discrepancy in B_{max} values was reduced by Gpp(NH)p addition, showing that uncoupling favors ocaperidone binding. The fact that ocaperidone only binds to a fraction of h5-HT_{1D}R when it is coexpressed with $G\alpha_{i1}\beta_1\gamma_2$ indicates that a substantial amount of h5-HT_{1D}R couples to $G\alpha_{i1}\beta_1\gamma_2$ in the absence of agonist. Thus, the significant level of constitutive activity of h5-HT_{1D}R was also demonstrated in this experiment. The [^{3}H]ocaperidone concentration binding data, together with the [^{3}H]5-HT competition binding experiments (Fig. 3), suggest the existence of a receptor conformation with high ocaperidone affinity (K_d of 0.5 nM) presumably associated with the noncoupled receptor and a conformation with low ocaperidone affinity (K_d of about 100 nM, not seen in the concentration binding experiment) associated with the coupled receptor.

Analyzing in more detail the curves obtained for the competition of [^{3}H]5-HT binding by ocaperidone (Fig. 3), a Hill coefficient of around unity was observed for membrane material containing the coupled receptor. This is indicative of a competitive inhibition mode. Analysis of the competition curves when using noncoupled receptor material gave a more complicated picture. In this case, the shallow curve for the inhibition of [^{3}H]5-HT by ocaperidone is inconsistent with competitive inhibition. The inhibition curve can be analyzed with a two-site binding model; in this case a 40%/60% distribution of "high" and "low" affinity inverse agonist binding sites are found. We do not think that this apparent dual set of binding sites can be explained by the occurrence of a fraction of the receptor being coupled to endogenous G-proteins. Indeed, such a coupled fraction would at most represent 15% of the receptor population (see above). Therefore we opt for explaining the shallow inhibition curve by assuming a complex mode of inhibition inconsistent with competitive inhibition. Data from [^{3}H]5-HT concentration binding experiments performed in the presence of various ocaperidone concentrations (Fig. 6) confirm that ocaperidone competes [^{3}H]5-HT binding in a competitive way at the coupled receptor, but at the noncoupled receptor another mode of inhibition appears. Barr and Manning (1997b) have already described the fact that one ligand (spiperone) can mediate different types of antagonism at a receptor (5-HT_{1A}R), depending on its affinity state. Regardless of which model can explain our observations, we clearly illustrated the complete different behavior of the inverse agonist ocaperidone depending on the conformation of h5-HT_{1D}R. Ocaperidone seems to drive the noncoupled h5-HT_{1D}R to a very stable Oca·R complex, which cannot be reverted by concentrations of 5-HT up to 20 nM. Stabilization of specific ligand-receptor complexes by inverse agonists is an idea that that has gained growing support in the literature (Kobilka, 1990; Bouaboula et al., 1997; Gether et al., 1997; Gether and Kobilka, 1998). A scheme integrating our observations and based on the two-state model of GPCR activation is presented in Fig. 7.

In conclusion, we have reconstituted a functional h5-HT_{1D}*R*-G-protein coupling in Sf9 cells. The receptor, when coexpressed with $G\alpha_{i1}\beta_1\gamma_2$, showed a high degree of constitutive activity. We could prove that an inverse agonist at h5-HT_{1D}R stabilizes a noncoupled receptor conformation. This expression system is very valuable for the evaluation of the behavior of ligands at specific receptors, especially for h5-HT_{1D}R where identification and use of inverse agonists might be important for the design of therapeutic strategies.

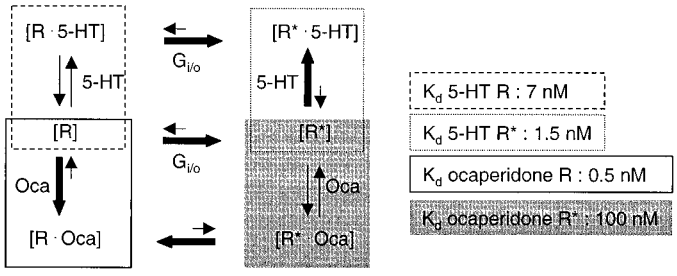


Fig. 7. h5-HT_{1D}R: hypothesis on the conformational changes upon G-protein coupling or ligand binding. The model presented integrates the observed effects and is based on the two-state model for GPCR activation. The thickness of the arrows indicates the direction toward which the equilibrium is likely to be driven. H5-HT_{1D}R has a high constitutive activity, i.e., it shows a substantial agonist-independent conformational change from the inactive (R) to the active receptor state (R*) in the presence of $G\alpha_{i1}\beta_1\gamma_2$. The receptor in this state is able to enhance [^{35}S]GTPγS binding to the G-protein, which explains the high basal [^{35}S]GTPγS binding levels observed. The natural agonist 5-HT further stabilizes this active receptor conformation (R*·5-HT) in the presence of G-proteins and further increases [^{35}S]GTPγS binding to $G\alpha_{i1}\beta_1\gamma_2$. Gpp(NH)p diminishes the pool of G-proteins able to interact with h5-HT_{1D}R and enriches the receptor population in the inactive state (R·5-HT) which has a lower 5-HT affinity. The inverse agonist ocaperidone is clearly bound with higher affinity by the receptor expressed alone (R) as compared with the receptor coexpressed with $G\alpha_{i1}\beta_1\gamma_2$ (R*). Binding of ocaperidone on the active receptor (R*) diminishes the levels of [^{35}S]GTPγS bound. This suggests that ocaperidone induces a receptor state that cannot activate the G-proteins (R·Oca). Apparent equilibrium dissociation constants for 5-HT and ocaperidone for the inactive (R) and active (R*) receptor states, as determined in our experiments, are shown.

Acknowledgments

We thank E. Le Poul for stimulating discussions and suggestions and C. Jolink for technical assistance. We also thank Drs. A. Gilman, J. Garrisson, and T. Haga for generously providing recombinant baculovirus strains for expression of diverse G-proteins.

References

Barker EL, Westphal RS, Schmidt D and Sanders-Bush E (1994) Constitutively active 5-hydroxytryptamine2C receptors reveal novel inverse agonist activity of receptor ligands. *J Biol Chem* **269:**11687–11690.

Barr AJ, Brass LF and Manning DR (1997a) Reconstitution of receptors and GTP-binding regulatory proteins (G proteins) in Sf9 cells: A direct evaluation of selectivity in receptor·G protein coupling. *J Biol Chem* **272:**2223–2229.

Barr AJ and Manning DR (1997b) Agonist-independent activation of Gz by the 5-hydroxytryptamine1A receptor co-expressed in *Spodoptera frugiperda* cells: Distinguishing inverse agonists from neutral antagonists. *J Biol Chem* **272:**32979–32987.

Bonaventure P, Schotte A, Cras P and Leysen JE (1997) Autoradiographic mapping of 5-HT1B- and 5-HT1D receptors in human brain using [^{3}H]alniditan, a new radioligand. *Receptors Channels* **5:**225–230.

Bonaventure P, Voorn P, Luyten WH, Jurzak M, Schotte A and Leysen JE (1998) Detailed mapping of serotonin 5-HT$_{1B}$ and 5-HT$_{1D}$ receptor messenger RNA and ligand binding sites in guinea-pig brain and trigeminal ganglion: Clues for function. *Neuroscience* **82:**469–484.

Bouaboula M, Perrachon S, Milligan L, Canat X, Rinaldi-Carmona M, Portier M, Barth F, Calandra B, Pecceu F, Lupker J, Maffrand JP, Le Fur G and Casellas P (1997) A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor: 1. Evidence for a new model of receptor/ligand interactions. *J Biol Chem* **272:**22330–22339.

Butkerait P, Zheng Y, Hallak H, Graham TE, Miller HA, Burris KD, Molinoff PB and Manning DR (1995) Expression of the human 5-hydroxytryptamine1A receptor in Sf9 cells: Reconstitution of a coupled phenotype by co-expression of mammalian G protein subunits. *J Biol Chem* **270:**18691–18699.

Clawges HM, Depree KM, Parker EM and Graber SG (1997) Human 5-HT1 receptor subtypes exhibit distinct G protein coupling behaviours in membranes from Sf9 cells. *Biochemistry* **36:**12930–12938.

Clitherow JW, Scopes DI, Skingle M, Jordan CC, Feniuk W, Campbell IB, Carter MC, Collington EW, Connor HE, Higgins GA, Beattie D, Kelly HA, Mitchell WL, Oxford AW, Wadsworth AH and Myers MB (1994) Evolution of a novel series of [(*N*,*N*-dimethylamino)propyl]- and piperazinylbenzanilides as the first selective 5-HT1D antagonists. *J Med Chem* **37:**2253–2257.

Gaster LM, Blaney FE, Davies S, Duckworth DM, Ham P, Jenkins S, Jennings AJ, Joiner GF, King FD, Mulholland KR, Wyman PA, Hagan JJ, Hatcher J, Jones BJ, Middlemiss DN, Price GW, Riley G, Roberts C, Routledge C, Selkirk J and Slade PD (1998) The selective 5-HT1B receptor inverse agonist 1′-methyl-5-[[2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl]-2,3,6,7-tetrahydrospiro[furo[2,3-*f*]indole-3,4′-piperidine] (SB-224289) potently blocks terminal 5-HT autoreceptor function both in vitro and in vivo. *J Med Chem* **41:**1218–1235.

Gether U, Ballesteros JA, Seifert R, Sanders-Bush E, Weinstein H and Kobilka BK (1997) Structural instability of a constitutive G protein-coupled receptor. *J Biol Chem* **272:**2587–2590.

Gether U and Kobilka BK (1998) G protein-coupled receptors. II. Mechanism of agonist activation. *J Biol Chem* **273:**17979–17982.

Grünewald S, Reilander H and Michel H (1996) In vivo reconstitution of dopamine D2S receptor-mediated G protein activation in baculovirus-infected insect cells: Preferred coupling to Gi1 versus Gi2. *Biochemistry* **35:**15162–15173.

Halazy S, Lamothe M and Jorand-Lebrun C (1997) 5-HT1B/1D antagonists and depression. *Exp Opin Ther Patents* **7:**339–352.

Hamblin MW and Metcalf MA (1991) Primary structure and functional characterization of a human 5-HT1D-type serotonin receptor. *Mol Pharmacol* **40:**143–148.

Hamel E, Gregoire L and Lau B (1993) 5-HT1 receptors mediating contraction in bovine cerebral arteries: A model for human cerebrovascular "5-HT1D beta" receptors. *Eur J Pharmacol* **242:**75–82.

Hanf R, Li Y, Szabo G and Fischmeister R (1993) Agonist-independent effects of muscarinic antagonists on Ca^{2+} and K^+ currents in frog and rat cardiac cells. *J Physiol* **461:**743–765.

Hartman JL IV and Northup JK (1996) Functional reconstitution in situ of 5-hydroxytryptamine2c (5HT2c) receptors with Galphaq and inverse agonism of 5HT2c receptor antagonists. *J Biol Chem* **271:**22591–22597.

Hepler JR, Kozasa T, Smrcka AV, Simon MI, Rhee SG, Sternweis PC and Gilman AG (1993) Purification from Sf9 cells and characterization of recombinant Gq alpha and G11 alpha: Activation of purified phospholipase C isozymes by G alpha subunits. *J Biol Chem* **268:**14367–14375.

Hoyer D and Martin G (1997) 5-HT receptor classification and nomenclature: Toward a harmonisation with the human genome. *Neuropharmacology* **36:**419–428.

Hoyer D and Schoeffter P (1988) 5-HT1D receptor-mediated inhibition of forskolin-stimulated adenylate cyclase activity in calf substantia nigra. *Eur J Pharmacol* **147:**145–147.

Iñiguez-Lluhi JA, Simon MI, Robishaw JD and Gilman AG (1992) G protein beta gamma subunits synthesized in Sf9 cells: Functional characterization and the significance of prenylation of gamma. *J Biol Chem* **267:**23409–23417.

Kelleher DJ and Johnson GL (1988) Transducin inhibition of light-dependent rhodopsin phosphorylation: Evidence for beta gamma subunit interaction with rhodopsin. *Mol Pharmacol* **34:**452–460.

Kobilka BK (1990) The role of cytosolic and membrane factors in processing of the human beta-2 adrenergic receptor following translocation and glycosylation in a cell-free system. *J Biol Chem* **265:**7610–768.

Leopoldt D, Harteneck C and Nurnberg B (1997) G proteins endogenously expressed in Sf 9 cells: Interactions with mammalian histamine receptors. *Naunyn-Schmiedebergs Arch Pharmakol* **356:**216–224.

Lesage AS, Wouters R, Van Gompel P, Heylen L, Vanhoenacker P, Haegeman G, Luyten WH and Leysen JE (1998) Agonistic properties of alniditan, sumatriptan and dihydroergotamine on human 5-HT1B and 5-HT1D receptors expressed in various mammalian cell lines. *Br J Pharmacol* **123:**1655–1665.

Leysen JE, Janssen PM, Gommeren W, Wynants J, Pauwels PJ and Janssen PA (1992) In vitro and in vivo receptor binding and effects on monoamine turnover in rat brain regions of the novel antipsychotics risperidone and ocaperidone. *Mol Pharmacol* **41:**494–508.

Leysen JE, Gommeren W, Heylen L, Luyten WH, Van de Weyer I, Vanhoenacker P, Haegeman G, Schotte A, Van Gompel P, Wouters R and Lesage AS (1996) Alniditan, a new 5-hydroxytryptamine1D agonist and migraine-abortive agent: Ligand-binding properties of human 5-hydroxytryptamine1D alpha, human 5-hydroxytryptamine1D beta, and calf 5-hydroxytryptamine1D receptors investigated with [^{3}H]5-hydroxytryptamine and [^{3}H]alniditan. *Mol Pharmacol* **50:**1567–1580.

Mewes T, Dutz S, Ravens U and Jakobs KH (1993) Activation of calcium currents in cardiac myocytes by empty beta-adrenoceptors. *Circulation* **88:**2916–2922.

Moskowitz MA (1992) Neurogenic versus vascular mechanisms of sumatriptan and ergot alkaloids in migraine. *Trends Pharmacol Sci* **13:**307–311.

Pauwels PJ (1997) 5-HT1B/D receptor antagonists. *Gen Pharmacol* **29:**293–303.

Peroutka S (1994) Pharmacological differentiation of human 5-HT1B and 5-HT1D receptors. *J Biol Signals* **3:**217–222.

Price GW, Burton MJ, Collin LJ, Duckworth M, Gaster L, Gothert M, Jones BJ, Roberts C, Watson JM and Middlemiss DN (1997) SB-216641 and BRL-15572–compounds to pharmacologically discriminate h5-HT1B and h5-HT1D receptors. *Naunyn-Schmiedebergs Arch Pharmakol* **356:**312–320.

Quehenberger O, Prossnitz ER, Cochrane CG, and Ye RD (1992) Absence of G(i) proteins in the Sf9 insect cell. Characterization of the uncoupled recombinant *N*-formyl peptide receptor. *J Biol Chem* **267:**19757–19760.

Thomas DR, Faruq SA, Balcarek JM and Brown AM (1995) Pharmacological characterisation of [^{35}S]-GTPgammaS binding to Chinese hamster ovary cell membranes stably expressing cloned human 5-HT1D receptor subtypes. *J Recept Signal Transduct Res* **15:**199–211.

Weinshank RL, Zgombick JM, Macchi MJ, Branchek TA and Hartig PR (1992) Human serotonin 1D receptor is encoded by a subfamily of two distinct genes: 5-HT1D alpha and 5-HT1D beta. *Proc Natl Acad Sci USA* **89:**3630–3634.

Wenzel-Seifert K, Hurt CM and Seifert R (1998) High constitutive activity of the human formyl peptide receptor. *J Biol Chem* **273:**24181–24189.

Wurch T, Colpaert FC and Pauwels PJ (1998) Chimeric receptor analysis of the ketanserin binding site in the human 5-Hydroxytryptamine1D receptor: Importance of the second extracellular loop and fifth transmembrane domain in antagonist binding. *Mol Pharmacol* **54:**1088–1096.

Zgombick JM, Borden LA, Cochran TL, Kucharewicz SA, Weinshank RL and Branchek TA (1993) Dual coupling of cloned human 5-hydroxytryptamine1D alpha and 5-hydroxytryptamine1D beta receptors stably expressed in murine fibroblasts: Inhibition of adenylate cyclase and elevation of intracellular calcium concentrations via pertussis toxin-sensitive G protein(s). *Mol Pharmacol* **44:**575–582.

Send reprint requests to: Josée Leysen, Janssen Research Foundation, Turnhoutseweg 30, B-2340 Beerse, Belgium. E-mail: jleysen2@janbe.jnj.com.