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Human 5-Hydroxytryptamine$_{5A}$ Receptors Activate Coexpressed G_i and G_o Proteins in *Spodoptera frugiperda* 9 Cells

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ABSTRACT

The ability of the human 5-hydroxytryptamine serotonin type 5A (h5-ht$_{5A}$) receptor to couple to G proteins from distinct families was investigated through the simultaneous infection of *Spodoptera frugiperda* 9 insect cells with recombinant baculoviruses encoding the various proteins. Expression of G proteins was demonstrated in immunoblots. Receptor-G protein coupling was monitored by high-affinity agonist binding and agonist-induced stimulation of [^{35}S]guanosine-5′-*O*-(3-thio) triphosphate binding to membranes. Receptors expressed alone displayed low-affinity agonist binding, and endogenous G proteins were only poorly stimulated on the addition of 5-hydroxytryptamine. When receptors were coexpressed with mammalian G_i/G_o proteins ($G\alpha_i$ or $G\alpha_o$ plus $G\beta_1\gamma_2$), the coupled phenotype was achieved: agonists bound with high affinity in a guanosine-5′-(β,γ-imido)triphosphate-sensitive manner and stimulated [^{35}S]guanosine-5′-*O*-(3-thio)triphosphate binding to high levels. These effects were not observed on coexpression with G_z/G_s/$G_{q/11/16}$ or $G_{12/13}$. Various ligands were evaluated for their agonistic, antagonistic, or inverse agonistic behavior in both receptor binding and activation assays. Although G_o displayed different receptor coupling characteristics than G_i proteins, no clear coupling preference was evident. Coexpression of receptors and $G\alpha_i$ subunits without $G\beta_1\gamma_2$ produced increases in both agonist affinity and maximum G protein activation that were smaller than those in the presence of $G\beta_1\gamma_2$, suggesting that $G\beta_1\gamma_2$ coexpression improves receptor-G protein coupling. Similarly, coexpression of receptors with $G\beta_1\gamma_2$ alone resulted in an improved interaction with endogenous G proteins. Our results demonstrate that h5-ht$_{5A}$ receptors expressed in *Spodoptera frugiperda* 9 cells selectively and functionally couple to coexpressed mammalian G_i and G_o proteins.

5-Hydroxytryptamine (5-HT) is a neurotransmitter that affects diverse physiological processes, including sleep, sexual behavior, food intake, locomotion, and mood. Schizophrenia, depression, and migraine are among the pathological conditions that are associated with a dysfunction of 5-HT transmission. At least 13 different 5-HT receptors have been identified to date. They belong to the superfamily of seven-transmembrane-domain receptors that couple to heterotrimeric guanine nucleotide-binding proteins (G proteins), with the exception of the 5-ht$_3$ receptor, which forms a 5-HT-gated ion channel (for review, Saudou and Hen, 1994; Hoyer and Martin, 1997).

The 5-ht$_{5A}$ and 5-ht$_{5B}$ receptors of the 5-ht$_5$ receptor subfamily were first identified in mice (Plassat et al., 1992; Matthes et al., 1993) and subsequently in rats (Erlander et al., 1993). Rees et al. (1994) cloned the human 5-ht$_{5A}$ receptor (h5-ht$_{5A}$) homolog, but a 5-ht$_{5B}$ receptor does not seem to be functionally expressed in humans (Rees et al., 1994). The physiological function of 5-ht$_5$ receptors is still unclear, partly due to a lack of specific ligands. Recently, results obtained with transgenic mice lacking the 5-ht$_{5A}$ receptor gene suggested the involvement of the receptor subtype in exploratory behavior (Grailhe et al., 1999). The mouse, rat, and human 5-ht$_5$ receptors have already been expressed in various cell lines. Initially, no effects on signal transduction systems could be demonstrated (Erlander et al., 1993; Matthes et al., 1993), although agonist binding to the recombinant receptor was found to be guanine nucleotide-sensitive

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; 5-CT, 5-carboxamidotryptamine; 5-MT, 5-methoxytryptamine; DHE, dihydroergotamine; E_{max}, relative maximum stimulation; G protein, guanine nucleotide-binding protein; $G_{i/o}$, combination of G_{i1}, G_{i2}, G_{i3}, and G_o proteins; Gpp(NH)p, guanosine-5′-(β,γ-imido)triphosphate; Gα, G protein α-subunit; G$\beta_1\gamma_2$, G protein $\beta_1\gamma_2$ dimer; h5-ht$_{5A}$, human 5-hydroxytryptamine type 5A; HEK, human embryonic kidney; IC$_{50}$-corr, corrected IC$_{50}$; I_{max}, relative maximum inhibition; LSD, lysergic acid diethylamide; m.o.i., multiplicity of infection; *Sf*9, *Spodoptera frugiperda* 9.

(Plassat et al., 1992). Negative coupling to adenylate cyclase activity was first reported for the rat 5-ht$_{5A}$ receptor expressed in C6 glioma cells (Carson et al., 1996). Recently, agonist-induced inhibition of adenylate cyclase activity was also demonstrated for the human 5-ht$_{5A}$ receptor expressed in human embryonic kidney (HEK) 293 cells (Francken et al., 1998; Hurley et al., 1998). In studies of agonist-induced stimulation of [^{35}S]guanosine-5′-*O*-(3-thio)triphosphate ([^{35}S]GTPγS) binding, h5-ht$_{5A}$ receptors expressed in HEK 293 cells were shown to couple to pertussis toxin-sensitive G proteins (Francken et al., 1998).

The *Spodoptera frugiperda* 9 (*Sf*9) insect cell/baculovirus system has already been successfully used to reconstitute the interaction of various G protein-coupled receptors with their cognate G proteins (Butkerait et al., 1995; Grünewald et al., 1996; Barr et al., 1997). When expressed in *Sf*9 cells at high levels, heterologous receptors display a predominantly uncoupled phenotype in the absence of recombinant G proteins due to the low background of endogenous G proteins (Butkerait et al., 1995; Boundy et al., 1996; Ohtaki et al., 1998). Therefore, receptor-G protein coupling specificity can be examined by coexpression in *Sf*9 cells of the receptor proteins with a series of G protein subtypes, through simultaneous infection with the appropriate recombinant baculoviruses. Successful receptor-G protein interaction is characterized by high-affinity and guanine nucleotide-sensitive agonist binding and by receptor-mediated activation of G proteins, as measured by agonist-stimulated [^{35}S]GTPγS binding or GTPase activity.

To evaluate the G protein-coupling profile of the h5-ht$_{5A}$ receptor in detail, we coexpressed combinations of receptor, G protein $\beta_1\gamma_2$ dimer (G$\beta_1\gamma_2$), and various G protein α-subunits (Gα subunits) in *Sf*9 insect cells. We measured the receptor coupling to members of each of the four families of G proteins using radioligand binding and [^{35}S]GTPγS binding to membranes and investigated the pharmacological properties of various 5-HT receptor ligands. It was found that the h5-ht$_{5A}$ receptor selectively couples to G$\alpha_{i/o}$ proteins and that coexpression of the G$\beta_1\gamma_2$ dimer facilitates receptor-G protein coupling.

Experimental Procedures

Materials. *Sf*9 insect cells were obtained from Invitrogen (Groningen, The Netherlands). The baculovirus transfer vector pAcGP67A and the BaculoGold DNA were purchased from PharMingen (San Diego, CA). The transfer vector pBacPAK9 was obtained from Clontech Laboratories (Palo Alto, CA). [^{3}H]5-Carboxamidotryptamine (5-CT; 50–100 Ci/mmol), [^{35}S]GTPγS (>1000 Ci/mmol), and the chemiluminescent Western detection kit (ECL-Plus) were purchased from Amersham Pharmacia Biotech (Little Chalfont, UK). 5-HT, 5-methoxytryptamine (5-MT), and dihydroergotamine (DHE) were purchased from Acros Organics (Geel, Belgium). Lysergic acid diethylamide (LSD) was obtained from Kenija Industriji (Yugoslavia). 5-CT was obtained from Research Biochemicals Inc. (Natick, MA). Methiothepin was purchased from Hoffman-La Roche (Basel, Switzerland). Pargyline was purchased from Sigma-Aldrich (St. Louis, MO). Grace's supplemented insect cell culture medium, Sf-900 II serum-free insect cell culture medium, and antibiotic/antimycotic solution were obtained from Life Technologies (Gaithersburg, MD). Fetal bovine serum was purchased from BioWhittaker (Walkersville, MD). The protein assay kit and the protein molecular weight marker were obtained from Bio-Rad Laboratories (Hercules, CA). Guanosine-5′-(β,γ-imido)triphosphate (Gpp(NH)p) and GDP were obtained from Boehringer-Mannheim (Mannheim, Germany). The anti-G$\alpha_{i/o/t/z/s}$ rabbit antiserum was purchased from Calbiochem (La Jolla, CA). The rabbit antisera for G$\alpha_{q/11}$, Gα_{12}, and Gα_{13} were obtained from Chemicon International (Temecula, CA). The goat antiserum for Gα_{16} was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The peroxidase-conjugated anti-rabbit and anti-goat secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PE).

5-HT, 5-CT, and 5-MT were dissolved and diluted in assay buffer. DHE, LSD, and methiothepin were dissolved and diluted in DMSO; the last 20-fold dilution step was performed in assay buffer. The dilution in the assay mixture was 10-fold. In all control assays, DMSO was added to a final concentration of 0.5%.

Baculoviruses containing cDNA-encoding rat Gα_{i1}, Gα_{i2}, Gα_{i3}, and Gα_o subunits were gifts from Dr. J. Garrison (University of Virginia) (Graber et al., 1992). The baculovirus for human Gα_z was a gift from Dr. D. Manning (University of Pennsylvania) (Butkerait et al., 1995). Baculoviruses for bovine G$\alpha_{s\text{-short-B}}$, mouse G$\alpha_q$, mouse G$\alpha_{11}$, and human G$\alpha_{16}$ were gifts from Dr. A. Gilman and Dr. T. Kozasa (University of Texas) (Hepler et al., 1993; Kozasa et al., 1993; Linder et al., 1993). Baculoviruses for mouse Gα_{12} and Gα_{13} were gifts from Dr. D. Dhanasekaran (Temple University, PA). The bovine G$\beta_1\gamma_2$ transfer vector was a gift from Dr. T. Haga (University of Tokyo, Japan) (Nakamura et al., 1995).

Cloning of h5-ht$_{5A}$ Receptor cDNA. The coding region of the human 5-ht$_{5A}$ receptor was amplified from a QuickScreen cDNA library (Clontech) by polymerase chain reaction using primers 5′-GCGATATGGACCCAGAGATGGATTTACCAGTGAACC-3′ and 5′-GCCTCGAGCCTCAGTGTTGCCTAGAAAAGAAGTTCTTG-3′. The inclusion of restriction sites (*Eco*RV and *Xho*I) within the oligonucleotide primers allowed cloning of the polymerase chain reaction fragment into the pcDNA3 vector (Invitrogen). The sequence of the insert was identical to that reported by Hurley et al. (1998) and contained a single silent mutation (T to C at nucleotide 300, counting from the A of the start codon), compared with the sequence deposited in the GenBank/EMBL database (accession numbers X81411 and X81412) by Rees et al. (1994).

Construction of Recombinant Transfer Vector. The h5-ht$_{5A}$ cDNA clone in pcDNA3 was digested with *Pst*I, blunt-ended with Klenow DNA polymerase, and digested with *Xba*I, yielding a 1145-bp fragment encoding the h5-ht$_{5A}$ receptor. This fragment was subcloned into the *Bam*HI (filled in with Klenow DNA polymerase) and *Xba*I positions of the multiple cloning site of the baculovirus transfer vector pAcGP67A, such that the gp67 signal sequence was fused in frame to the N terminus of the h5-ht$_{5A}$ coding sequence via a nine-amino acid linker sequence (gp67-ADRCDMDPE-h5-ht$_{5A}$). For the pBacPAK9-based transfer vector, the h5-ht$_{5A}$ cDNA was excised from the pcDNA3 clone by digestion with *Eco*RI and *Xho*I. The 1142-bp fragment encoding the h5-ht$_{5A}$ receptor was subcloned into the multiple cloning site of the transfer vector pBacPAK9 that was digested with the same restriction enzymes. Protein expression was under control of the polyhedrin promoter in both transfer vectors. The DNA insert sequences were confirmed by sequencing both strands of the double-stranded DNA.

Generation of Recombinant Baculoviruses. Transfer of the h5-ht$_{5A}$ receptor cDNA into the wild-type *Autographa californica* nuclear polyhedrosis virus genome was accomplished by homologous recombination. *Sf*9 insect cells were cotransfected with linearized modified *A. californica* nuclear polyhedrosis virus baculovirus DNA (BaculoGold) and the h5-ht$_{5A}$-containing recombinant transfer vector using standard techniques (O'Reilly et al., 1992). Purification of recombinant viruses, amplification of purified virus stocks, and determination of virus titers were performed as described by O'Reilly et al. (1992).

Insect Cell Culture and Baculovirus Infection. *Sf*9 cells were grown at 27°C and at an ambient atmosphere in suspension culture using spinner flasks or in monolayers. For viral stock production, Grace's insect cell culture medium was used supplemented with 10%

fetal bovine serum, 0.2 mM L-glutamine, and 1% antibiotic/antimycotic solution, whereas Sf-900 II serum-free insect cell culture medium, supplemented with 0.2 mM L-glutamine and 1% antibiotic/antimycotic solution, was used in recombinant protein expression experiments. Cell viability was determined by trypan blue staining. Cells (50–500 ml) at a density of 1×10^6 cells/ml (log phase growth) were infected with a h5-ht$_{5A}$ receptor-encoding baculovirus at a multiplicity of infection (m.o.i.) of 2 (unless stated otherwise), with a G$\beta_1\gamma_2$-encoding virus (m.o.i. = 1) and/or with a Gα-encoding virus (m.o.i. = 2–4). For the expression of single Gα subunits, the m.o.i. was 4 for any Gα baculovirus, whereas for the expression of multiple Gα subunits (Gα_{i1}, Gα_{i2}, Gα_{i3}, and Gα_o, abbreviated as G$\alpha_{i/o}$) the m.o.i. was 2 for each virus. At 48 h postinfection, cells were harvested by centrifugation (10 min at 2000*g* at 4°C), washed with ice-cold PBS, and stored at −80°C or used directly for membrane preparation.

Membrane Preparation and Determination of Protein Content. Harvested *Sf*9 cells were washed with ice-cold 50 mM Tris-HCl buffer, pH 7.4; resuspended in hypotonic 10 mM Tris-HCl buffer, pH 7.4; and homogenized with an UltraTurrax homogenizer (Janke and Kunkel, Staufen, Germany) for 5 s. The homogenate was centrifuged at 30,000*g* for 20 min at 4°C. The membrane pellet was resuspended in 50 mM Tris-HCl buffer, pH 7.4, containing 10% glycerol and stored in aliquots at −80°C. Protein content in membrane preparations was estimated with the Bradford protein assay (Bradford, 1976), using the Bio-Rad kit. BSA was used as a standard.

Immunoblot Analysis. Membrane protein (1, 4, or 10 μg) was incubated in 62.5 mM Tris-HCl buffer, pH 6.8, containing 10% glycerol, 5% SDS, and 0.01% bromophenol blue at 37°C for 2 h. Proteins were separated by SDS-polyacrylamide gel electrophoresis and were transferred to polyvinylidene-difluoride membranes, using standard techniques. Immunodetection of Gα subunits was performed with 1:1000 dilutions of the G$\alpha_{i/o/t/z/s}$, G$\alpha_{q/11}$, Gα_{16}, Gα_{12}, and Gα_{13} antisera. The peroxidase-conjugated anti-rabbit and anti-goat secondary antibodies were diluted 1:5000. Bands were visualized by chemiluminescence using the ECL-Plus detection kit.

Radioligand Binding. [^{3}H]5-CT binding experiments were performed essentially as described previously (Francken et al., 1998). Briefly, 6 μg of membrane protein was diluted in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM $MgCl_2$, 1 mM EGTA, and 10 μM pargyline and incubated with [^{3}H]5-CT for 1 h at 25°C in a volume of 0.5 ml. Nonspecific binding was estimated in the presence of 10 μM methiothepin. Reactions were terminated by rapid filtration through glass fiber (GF/B) filters (Whatman, Kent, UK) presoaked in 0.1% polyethyleneimine using a Brandel (Gaithersburg, MD) 96-sample harvester. Filters were washed twice, and filter-bound radioactivity was counted in a liquid scintillation spectrometer (Tricarb) using scintillation fluid (Ultima Gold MV; Packard Instrument Company, Meriden, CT). For radioligand concentration-binding isotherms, 12 concentrations of [^{3}H]5-CT, in a range of 0.1 to 25 nM, were used. Competition binding experiments were performed using 2 nM [^{3}H]5-CT; compounds were added at 7 to 12 concentrations.

[^{35}S]GTPγS Binding. [^{35}S]GTPγS binding experiments were performed as previously described (Francken et al., 1998). Briefly, 12 μg of membrane protein was diluted in 50 mM Tris-HCl buffer, pH 7.4, containing 50 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 0.1 mM dithiothreitol, 10 μM pargyline, and 1 μM GDP and preincubated with compound for 30 min at 30°C in a volume of 0.45 ml. Then, 50 μl of [^{35}S]GTPγS in assay buffer was added to a final concentration of 0.2 nM, and the assay mixtures were further incubated for 30 min at 30°C. Reactions were terminated by rapid filtration through GF/B filters, presoaked in assay buffer, using a 40-well manual filtration manifold or a Brandel 48-sample harvester. Filters were washed twice, and filter-bound radioactivity was counted in a liquid scintillation spectrometer. Basal [^{35}S]GTPγS binding was measured in the absence of compound. Compounds were added at 9 to 11 concentrations. Nonspecific [^{35}S]GTPγS binding, as measured in the presence of 100 μM GTPγS, did not exceed 10% of basal binding and was never subtracted from experimental data.

Data Analysis. Radioligand concentration-binding isotherms (rectangular hyperbola) were calculated by nonlinear regression analysis according to algorithms described by Oestreicher and Pinto (1987), and sigmoidal inhibition curves were calculated by nonlinear regression using the Prism program (GraphPad Software, San Diego, CA). B_{max} and K_d values of the radioligand and IC_{50} values of inhibitors were derived from the curve fitting.

Stimulation of [^{35}S]GTPγS binding was calculated as 100 times the difference between stimulated and basal binding (in cpm) divided by the amount of basal binding (in cpm). Agonist concentration-response curves and antagonist inhibition curves were analyzed by nonlinear regression using GraphPad Prism. EC_{50} and IC_{50} values were derived from the curves. IC_{50} values were corrected as follows: corrected IC_{50} (IC_{50}-corr) = $IC_{50}/\{1 + [5\text{-HT}]/EC_{50}(5\text{-HT})\}$. Relative maximum stimulation (E_{max}) values were calculated as percentage of the maximum stimulation obtained with 10 μM 5-HT, and relative maximum inhibition (I_{max}) values were calculated as percentage of the inhibition from maximum 5-HT (10 μM)-stimulated [^{35}S]GTPγS binding to basal level.

Statistical *F* tests and Student's *t* tests were performed, and all figures were prepared using GraphPad Prism.

Results

Expression of h5-ht$_{5A}$ Receptors and G Protein Subunits in *Sf*9 Insect Cells. The h5-ht$_{5A}$ receptor coding sequence was cloned from a cDNA library, and recombinant baculoviruses were generated and used to infect *Sf*9 cells. In preliminary [^{3}H]5-CT concentration-binding experiments on membranes of *Sf*9 cells infected at an m.o.i. of 3, higher expression levels were found for virus generated with the pAcGP67A transfer vector (B_{max} = 63 ± 11 pmol/mg protein, K_d = 10.1 ± 2.0 nM, mean ± S.D., n = 5) than for pBacPAK9-based virus (B_{max} = 23 ± 2 pmol/mg protein, K_d = 5.6 ± 1.0 nM, n = 3), probably due to the presence of the gp67 signal sequence. No specific [^{3}H]5-CT binding could be detected to membranes of uninfected or wild-type baculovirus-infected cells (data not shown). Further expression experiments were performed with the pAcGP67A-based virus at an m.o.i. of 2.

The effect of coexpression of various G protein subunits (m.o.i. = 1–4) on the affinity of [^{3}H]5-CT for the h5-ht$_{5A}$ receptor was determined using [^{3}H]5-CT concentration-binding experiments. Examples of [^{3}H]5-CT saturation curves are presented in Fig. 1. Table 1 summarizes the mean K_d and B_{max} values and shows the binding data for h5-ht$_{5A}$-HEK 293 cell membranes for comparison (Francken et al., 1998). All [^{3}H]5-CT concentration-binding isotherms were best fitted to a one-binding-site model compared with a two-binding-site model, using nonlinear regression (*F* test, $P > .05$). Receptors expressed alone in *Sf*9 cells yielded a B_{max} value of 39 ± 12 pmol/mg protein and a K_d value of 7.8 ± 0.9 nM, which is a K_d value similar to that of the low-affinity form of the receptor in h5-ht$_{5A}$-HEK 293 cells. Coexpression of receptors with Gβ_1 and Gγ_2 (G$\beta_1\gamma_2$ baculovirus, m.o.i. = 1) resulted in a slight, but statistically significant, increase in [^{3}H]5-CT affinity (Student's *t* test, $P < .05$) (Fig. 1A, Table 1), suggesting improved coupling of the recombinant receptors to endogenous G proteins. Coexpression of h5-ht$_{5A}$ receptors with Gα_{i1}, Gα_{i2}, or Gα_{i3} (m.o.i. = 4) or with a mixture of Gα_{i1}, Gα_{i2}, Gα_{i3}, and Gα_o (further designated as G$\alpha_{i/o}$; m.o.i. = 2 for each virus) also significantly increased [^{3}H]5-CT affinity (Student's *t* test, $P < .05$) (Fig. 1B), whereas no effect was ob-

served with Gα_o, Gα_z, Gα_s, Gα_{11}, Gα_{16}, Gα_{12}, or Gα_{13} subunits (Table 1). The small, but statistically significant, increase in [^{3}H]5-CT affinity that was observed on coexpression with Gα_q is considered as a spurious finding, considering the lack of a Gpp(NH)p effect on agonist binding (Table 1, see Fig. 4). When G$\beta_1\gamma_2$ subunits (m.o.i. = 1) were expressed in addition to Gα subunits and receptors, [^{3}H]5-CT affinities further increased for G$\alpha_{i/o}$, Gα_{i1}, Gα_{i2}, Gα_{i3}, and Gα_o (Student's t test, $P < .05$) (Fig. 1B) but not for Gα_z, Gα_s, Gα_q, Gα_{11}, Gα_{16}, Gα_{12}, or Gα_{13} (Table 1).

The expression of recombinant Gα subunits was verified by immunoblot analysis using commercially available antibodies. Figure 2 shows immunoblots for the membranes of *Sf*9 cells expressing receptor and mammalian G protein trimers. For the different Gα proteins, immunoreactivity was demonstrated in the respective experimental membranes. It should be noted that using the same antiserum directed against a common peptide sequence, the immunoreactivity for Gα_o was much stronger than that for the Gα_i subunits, suggesting higher Gα protein expression levels.

In the presence of the individual G$_i$ or G$_o$ trimers, the affinity of [^{3}H]5-CT was intermediate to that of the high- and low-affinity forms of the receptor in stably transfected HEK 293 cells (Francken et al., 1998). Only with the simultaneous expression of a mixture of G$_i$ and G$_o$ proteins (G$_{i/o}$) did the affinity of [^{3}H]5-CT equal that for the high-affinity state of the receptor, probably due to the occurrence of a lower receptor-to-G protein ratio. Indeed, the infection of *Sf*9 cells with baculoviruses encoding each of the four G protein subtypes resulted in a relatively low level of receptor binding sites (Table 1), and the high m.o.i. for the Gα protein-encoding baculoviruses implicates an increased overall number of G proteins (Fig. 2). Alternatively, coexpression with the mixture of G$_{i/o}$ proteins might mimic a more natural situation, in which the receptor is able to interact with all of the used G protein subtypes. It should be noted that a decrease in receptor number was not systematically observed on coexpression with G protein subunits. For example, coexpression with Gα_q and G$\beta_1\gamma_2$ resulted in a B_{max} value that was higher than when the receptor was expressed alone (Table 1). Differences in receptor expression levels between similar experiments were also observed by other groups (Butkerait et al., 1995) and are difficult to explain.

Pharmacological Characterization of h5-ht$_{5A}$ Receptors Expressed Alone or Coexpressed with G Protein Subunits. Various 5-HT receptor ligands were used to inhibit [^{3}H]5-CT binding to membranes of baculovirus-infected *Sf*9 insect cells. pIC$_{50}$ values were derived from inhibition curves and are summarized in Table 2. The pharmacological profile of h5-ht$_{5A}$ receptors expressed alone in *Sf*9 cells was different from that in stably transfected h5-ht$_{5A}$-HEK 293 cells; the agonists 5-CT, 5-HT, and 5-MT had 3.2- to 3.6-fold lower affinities (Student's t test, $P < .05$), whereas DHE, LSD, and methiothepin had slightly higher affinities for the receptor expressed alone in *Sf*9 cells. The rank order of potency of the tested compounds was LSD > methiothepin > 5-CT > DHE > 5-HT > 5-MT. This profile did not change on coexpression with G$\beta_1\gamma_2$ subunits, although agonist affinities were slightly, but never significantly, increased.

The simultaneous expression of receptor and G$\beta_1\gamma_2$ together with Gα_{i1}, Gα_{i2}, Gα_{i3}, Gα_o, or the mixture of G$\alpha_{i/o}$ subunits, but not together with G$_z$ or G$_s$, resulted in an increase in the agonist affinities; the pIC$_{50}$ values were very similar to those for h5-ht$_{5A}$-HEK 293 membranes (Student's t test, $P > .05$) (Table 2). Figure 3 compares the inhibition curves of the tested compounds for *Sf*9 cells expressing the h5-ht$_{5A}$ receptor alone and in combination with Gα_{i1} and G$\beta_1\gamma_2$. In contrast to the agonists, the affinity of methiothepin significantly decreased up to 1 log unit on coexpression of G$_i$/G$_o$ proteins. Decreases in DHE and LSD affinities were minor on coexpression of individual G$_i$ or G$_o$ proteins but appeared significant on coexpression of the mixture of G$_{i/o}$ proteins.

Effect of Gpp(NH)p on [^{3}H]5-CT Binding. The interaction of h5-ht$_{5A}$ receptors with endogenous or coexpressed G proteins in membranes of baculovirus-infected *Sf*9 cells was investigated by measuring the sensitivity of agonist binding to the addition of the nonhydrolyzable GTP analog

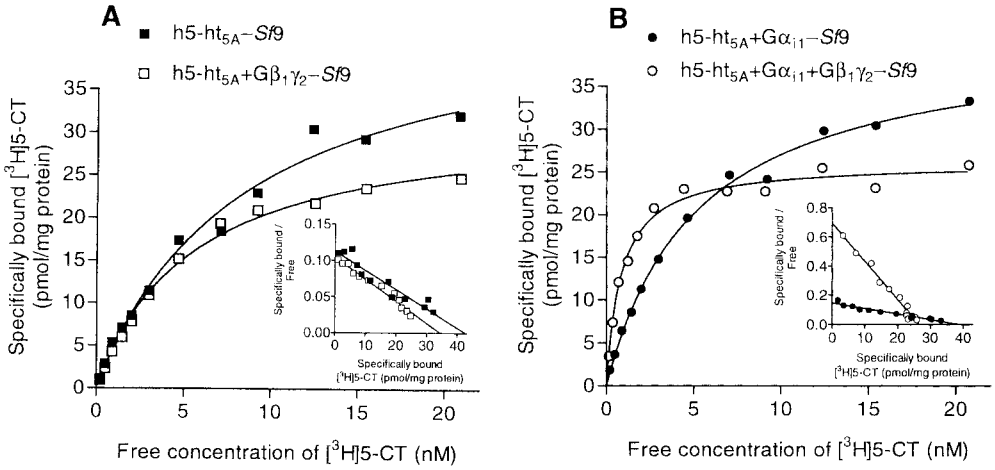

Fig. 1. Concentration-binding isotherms and Scatchard plots (insets) of specific [^{3}H]5-CT binding to membranes of baculovirus-infected *Sf*9 insect cells expressing h5-ht$_{5A}$ receptors alone (A, ■) or with Gβ_1 and Gγ_2 subunits (A, □) or coexpressing h5-ht$_{5A}$ receptors with Gα_{i1} alone (B, ●) or Gα_{i1} with Gβ_1 and Gγ_2 (B, ○). The data represent mean values of duplicate determinations from a typical experiment of three to six independent experiments. *Sf*9 cells were harvested after 48-h infection with a set of baculoviruses encoding the h5-ht$_{5A}$ receptor (m.o.i. = 2), Gα subunits (m.o.i. = 4), and/or G$\beta_1\gamma_2$ subunits (m.o.i. = 1). Radioligand binding studies were performed on membranes as described in *Experimental Procedures*. Isotherms were best fitted to a one-binding-site model using nonlinear regression analysis. B_{max} and K_d values were derived for each individual experiment, and mean values are summarized in Table 1.

Gpp(NH)p. [^{3}H]5-CT concentration-binding experiments in the presence and absence of 100 μM Gpp(NH)p were performed in parallel, and the K_d values were compared using a paired Student's t test (Table 1). Figure 4 visualizes the ratio of K_d values for [^{3}H]5-CT binding in the presence and absence of Gpp(NH)p. The affinity of [^{3}H]5-CT observed for the h5-ht$_{5A}$ receptor expressed alone was unaffected by Gpp(NH)p, suggesting the absence of interaction with endogenous G proteins. The small increase in affinity achieved by G$\beta_1\gamma_2$ coexpression was completely reversed by Gpp(NH)p. The affinity of [^{3}H]5-CT significantly decreased on Gpp(NH)p addition to membranes of *Sf*9 cells coexpressing the h5-HT$_{5A}$ receptor together with G$\beta_1\gamma_2$ and either the G$\alpha_{i/o}$ mixture, Gα_{i1}, Gα_{i2}, Gα_{i3}, or Gα_o, whereas no significant decrease in affinity was observed for the other G protein coexpressions (Fig. 4).

5-HT-Stimulated [^{35}S]GTPγS Binding. Receptor-mediated activation of G proteins was examined by concentration-dependent 5-HT-stimulated binding of [^{35}S]GTPγS to membranes of baculovirus-infected *Sf*9 cells. The activation of G proteins involves stimulation of GDP/GTP exchange at the Gα subunit and can be measured by the incorporation of the non-hydrolyzable GTP analog [^{35}S]GTPγS (Wieland and Jakobs, 1994). Figure 5 depicts the dose-dependent increase in 5-HT-stimulated [^{35}S]GTPγS binding for *Sf*9 cells expressing h5-ht$_{5A}$ receptors alone or together with G$\beta_1\gamma_2$ and/or the G$\alpha_{i/o}$ mixture. In membranes of *Sf*9 cells expressing only h5-ht$_{5A}$ receptors without mammalian G protein subunits, stimulation of the receptors with 5-HT resulted in an increase in [^{35}S]GTPγS binding to a maximum of 40% over basal, probably due to the activation of endogenous G proteins. Coexpression of G$\beta_1\gamma_2$ resulted in a significant increase of the maximum response (Student's t test, $P < .05$), up to 110% over basal. When the receptor was coexpressed with the mixture of G$\alpha_{i/o}$ subunits, without or with G$\beta_1\gamma_2$, the maximum stimulation was 330 and 570%, respectively. The effect of 5-HT was specific for the h5-ht$_{5A}$ receptor, because 5-HT did not affect [^{35}S]GTPγS binding to membranes from uninfected or wild-type baculovirus-infected *Sf*9 cells (data not shown).

Mean pEC$_{50}$ and maximum stimulation values for 5-HT, tested on a series of 26 receptor/G protein combinations expressed in *Sf*9 cells, are summarized in Table 3. Coexpressions with the individual Gα_{i1}, Gα_{i2}, Gα_{i3}, or Gα_o subunits yielded maximum responses that were comparable with those for the G$\alpha_{i/o}$ mixture. For the Gα_i subunits, additional coexpression of G$\beta_1\gamma_2$ markedly increased stimulation of [^{35}S]GTPγS binding, as observed for the G$\alpha_{i/o}$ mixture (Table 3). For Gα_o, however, coexpression with G$\beta_1\gamma_2$ significantly (Student's t test, $P < .05$) decreased the maximum stimula-

TABLE 1

K_d and B_{max} values for [^{3}H]5-CT binding to membranes of *Sf*9 cells coexpressing the h5-ht$_{5A}$ receptor and G protein subunits of the G$_{i/o}$, G$_s$, G$_{q/11}$, and G$_{12/13}$ family

Radioligand binding studies were performed, and K_d and B_{max} values were derived as described in *Experimental Procedures*. The results are mean ± S.D. values from n independent experiments.

Proteins Expressed	No Addition			+ 100 μM Gpp(NH)p		
	K_d	B_{max}	n	K_d	B_{max}	n
	nM	*pmol/mg protein*		*nM*	*pmol/mg protein*	
h5-ht$_{5A}$-HEK 293[a]	2.3 ± 0.7[c]	31 ± 4[c]	6	4.3 ± 0.9	29 ± 7	4
High	0.4 ± 0.2	8 ± 4				
Low	5.5 ± 2.0	25 ± 4				
h5-ht$_{5A}$-*Sf*9	7.8 ± 0.9	39 ± 12	3	7.1 ± 0.1	32 ± 8	3
h5-ht$_{5A}$ + G$\beta_1\gamma_2$-*Sf*9	5.6 ± 1.6[d]	29 ± 9	3	8.7 ± 1.1[f]	32 ± 10	3
h5-ht$_{5A}$ + G$\alpha_{i/o}$-*Sf*9[b]	3.7 ± 0.8[d]	16 ± 1	3	5.4 ± 0.8	15 ± 3	2
h5-ht$_{5A}$ + G$\alpha_{i/o}$ + G$\beta_1\gamma_2$-*Sf*9[b]	0.8 ± 0.2[e]	8 ± 2	4	6.9 ± 1.5[f]	5 ± 4	3
h5-ht$_{5A}$ + Gα_{i1}-*Sf*9	5.2 ± 1.7[d]	46 ± 9	5	4.6 ± 0.6	38 ± 7	3
h5-ht$_{5A}$ + Gα_{i1} + G$\beta_1\gamma_2$-*Sf*9	1.7 ± 0.7[e]	19 ± 3	6	5.9 ± 2.0[f]	19 ± 2	3
h5-ht$_{5A}$ + Gα_{i2}-*Sf*9	4.2 ± 1.1[d]	29 ± 6	5	5.4 ± 1.9	31 ± 14	3
h5-ht$_{5A}$ + Gα_{i2} + G$\beta_1\gamma_2$-*Sf*9	1.9 ± 0.5[e]	18 ± 3	6	7.5 ± 4.0[f]	19 ± 3	3
h5-ht$_{5A}$ + Gα_{i3}-*Sf*9	4.8 ± 1.0[d]	28 ± 8	5	5.1 ± 1.0	22 ± 2	3
h5-ht$_{5A}$ + Gα_{i3} + G$\beta_1\gamma_2$-*Sf*9	1.8 ± 0.6[e]	25 ± 6	6	6.0 ± 1.2[f]	30 ± 8	3
h5-ht$_{5A}$ + Gα_o-*Sf*9	8.0 ± 1.9	29 ± 5	4	8.0 ± 0.7	30 ± 5	4
h5-ht$_{5A}$ + Gα_o + G$\beta_1\gamma_2$-*Sf*9	2.0 ± 0.3[e]	42 ± 6	4	6.8 ± 0.7[f]	47 ± 8	4
h5-ht$_{5A}$ + Gα_z-*Sf*9	8.1 ± 0.9	26 ± 5	3	6.8 ± 1.9	24 ± 1	2
h5-ht$_{5A}$ + Gα_z + G$\beta_1\gamma_2$-*Sf*9	7.7 ± 1.8	24 ± 3	3	7.3 ± 0.3	24 ± 1	2
h5-ht$_{5A}$ + G$\alpha_{s\text{-}s}$-*Sf*9	7.2 ± 3.1	45 ± 3	5	6.4 ± 3.3	47 ± 4	3
h5-ht$_{5A}$ + G$\alpha_{s\text{-}s}$ + G$\beta_1\gamma_2$-*Sf*9	7.4 ± 2.1	34 ± 6	4	7.6 ± 1.9	30 ± 7	3
h5-ht$_{5A}$ + Gα_q-*Sf*9	5.6 ± 0.7[d]	67 ± 12	3	6.6 ± 0.4	68 ± 6	3
h5-ht$_{5A}$ + Gα_q + G$\beta_1\gamma_2$-*Sf*9	6.0 ± 0.9	51 ± 6	3	7.0 ± 1.8	59 ± 7	3
h5-ht$_{5A}$ + Gα_{11}-*Sf*9	6.6 ± 2.4	42 ± 9	4	6.5 ± 2.5	38 ± 9	4
h5-ht$_{5A}$ + Gα_{11} + G$\beta_1\gamma_2$-*Sf*9	5.0 ± 0.8	38 ± 9	3	4.5 ± 1.2	35 ± 12	3
h5-ht$_{5A}$ + Gα_{16}-*Sf*9	7.1 ± 0.8	58 ± 5	3	7.1 ± 1.0	55 ± 10	3
h5-ht$_{5A}$ + Gα_{16} + G$\beta_1\gamma_4$-*Sf*9	6.9 ± 1.8	57 ± 13	3	7.8 ± 1.0	60 ± 15	3
h5-ht$_{5A}$ + Gα_{12}-*Sf*9	7.7 ± 3.3	11 ± 1	4	8.9 ± 6.5	12 ± 3	3
h5-ht$_{5A}$ + Gα_{12} + G$\beta_1\gamma_2$-*Sf*9	10.7 ± 2.0	9 ± 2	3	8.5 ± 4.1	7 ± 2	2
h5-ht$_{5A}$ + Gα_{13}-*Sf*9	6.6 ± 2.6	27 ± 9	4	6.5 ± 2.6	27 ± 6	4
h5-ht$_{5A}$ + Gα_{13} + G$\beta_1\gamma_2$-*Sf*9	6.9 ± 1.6	24 ± 5	3	6.2 ± 1.6	23 ± 6	3

[a] Data for h5-ht$_{5A}$-HEK 293 were taken from Francken et al., 1998. High and low denote the K_d and B_{max} values of high- and low-affinity agonist binding site, respectively.
[b] G$\alpha_{i/o}$ represents the combination of Gα_{i1}, Gα_{i2}, Gα_{i3}, and Gα_o subunits.
[c] Values obtained from curve-fitting to a one-binding-site model, although a significantly better fit was achieved with a two-binding-site model (F test, $P < .005$).
[d] K_d value (for h5-ht$_{5A}$ receptors coexpressed with a Gα subunit or with G$\beta_1\gamma_2$ that is significantly (Student's t test, $P < .05$) different from the K_d value for h5-ht$_{5A}$-*Sf*9.
[e] K_d value (for h5-ht$_{5A}$ receptors coexpressed with a Gα subunit plus G$\beta_1\gamma_2$ that is significantly (Student's t test, $P < .05$) different from the K_d value for h5-ht$_{5A}$ + G$\beta_1\gamma_2$-*Sf*9.
[f] K_d value of [^{3}H]5-CT binding in the presence of 100 μM Gpp(NH)p that is significantly (Student's t test, $P < .05$) different from the K_d value in the absence of Gpp(NH)p.

tion of [^{35}S]GTPγS binding. It should be noted that the absolute values for basal [^{35}S]GTPγS binding (in cpm) were 2.6-fold higher for Gα$_o$/Gβ$_1$γ$_2$ than for Gα$_i$/Gβ$_1$γ$_2$ when coexpressed with h5-ht$_{5A}$ receptors, in contrast to coexpressions with Gα$_i$ or Gα$_o$, which showed comparable levels of agonist-independent [^{35}S]GTPγS binding (data not shown). For the coexpressions including Gα$_z$, Gα$_s$, Gα$_q$, Gα$_{11}$, and Gα$_{16}$, a small 5-HT-induced stimulation of [^{35}S]GTPγS binding was detected, but the maximum stimulation was never significantly higher than the appropriate control sample. No stimulation was observed for the Gα$_{12}$ and Gα$_{13}$ coexpressions.

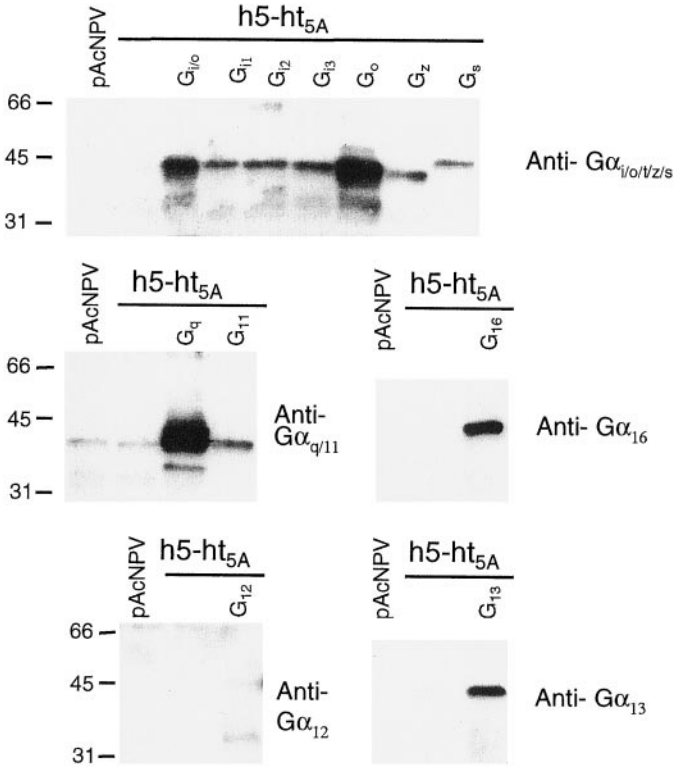

Fig. 2. Immunoblot analysis of baculovirus-infected *Sf*9 membranes using anti-Gα subunit antisera. The analysis was performed on membranes of *Sf*9 cells infected with wild-type baculovirus (pAcNPV) or a combination of recombinant baculoviruses encoding h5-ht$_{5A}$ receptor and/or G$_{i1}$, G$_{i2}$, G$_{i3}$, G$_o$, G$_z$, G$_s$, G$_q$, G$_{11}$, G$_{16}$, G$_{12}$, or G$_{13}$ heterotrimers (Gαβ$_1$γ$_2$), as indicated above each lane. The antisera that were used to visualize expression of the mammalian Gα proteins are indicated at the right of the bands, whereas the positions of the molecular weight marker proteins are indicated at the left. The anti-Gα$_{i/o/t/z/s}$ antiserum was tested on 4 μg of membrane protein, anti-Gα$_{q/11}$ antiserum was tested on 1 μg, and anti-Gα$_{16}$, anti-Gα$_{12}$, and anti-Gα$_{13}$ antisera were tested on 10 μg of membrane protein. G$_{i/o}$ represents the simultaneous expression of G$_{i1}$, G$_{i2}$, G$_{i3}$, and G$_o$ proteins.

Modulation of [^{35}S]GTPγS Binding by 5-HT Receptor Ligands. Several 5-HT receptor ligands were examined for their ability to modulate [^{35}S]GTPγS binding to membranes of *Sf*9 cells, expressing h5-ht$_{5A}$ receptors with Gβ$_1$γ$_2$ and either Gα$_{i1}$, Gα$_{i2}$, Gα$_{i3}$, or Gα$_o$. Figure 6 shows, as an example, the mean curves for the coexpression of h5-ht$_{5A}$ receptors with Gα$_{i1}$ and Gβ$_1$γ$_2$. Table 4 summarizes the pEC$_{50}$, E_{max}, pIC$_{50}$-corr, and I_{max} values from all [^{35}S]GTPγS dose-response and inhibition curves.

For each of the coexpressed combinations tested, 5-CT and 5-MT produced maximum responses similar to 5-HT, confirming their full agonistic properties (Francken et al., 1998). DHE and LSD stimulated [^{35}S]GTPγS binding to about 50% of the 5-HT level for the G$_i$ coexpressions (i.e., behaved as partial agonists), whereas for the G$_o$ coexpression, maximum stimulation approached the level of 5-HT (i.e., DHE and LSD behaved as full agonists). Methiothepin behaved as an inverse agonist as it inhibited [^{35}S]GTPγS binding to about −10% below its basal level (5-HT level set at 100%) for Gα$_{i1}$, Gα$_{i2}$, or Gα$_{i3}$ and to −24% below its basal level for Gα$_o$ (see Fig. 6 for Gα$_{i1}$; data not shown for Gα$_{i2}$, Gα$_{i3}$, and Gα$_o$). However, no reproducible curves could be derived from the methiothepin data points. The antagonistic properties of DHE, LSD, and methiothepin were investigated using [^{35}S]GTPγS binding to membranes of the same four coexpressions. DHE and LSD inhibited 5-HT (10 μM)-stimulated [^{35}S]GTPγS binding to the level of their own agonistic effect. Methiothepin behaved again as an inverse agonist, inhibiting [^{35}S]GTPγS binding below the basal level.

Discussion

Little is known about the pharmacological and functional properties of cloned 5-ht$_5$ receptors. Recently, h5-ht$_{5A}$ receptors were shown to mediate inhibition of adenylate cyclase

TABLE 2

Inhibition by various 5-HT receptor ligands of [^{3}H]5-CT (2 nM) binding to membranes of *Sf*9 cells coexpressing the h5-ht$_{5A}$ receptor and G protein subunits of the G$_{i/o}$ and G$_s$ family

Radioligand binding studies were performed as described in *Experimental Procedures*, and pIC$_{50}$ (−logM) values were derived from individual curves. Results are mean pIC$_{50}$ ± S.D. values from *n* independent experiments.

Proteins Expressed	pIC$_{50}$					
	5-CT	5-HT	5-MT	DHE	LSD	Methiothepin
	−logM (n)					
h5-ht$_{5A}$-HEK 293[a]	8.28 ± 0.04 (4)	7.40 ± 0.08 (7)	7.17 ± 0.08 (3)	7.15 ± 0.09 (4)	8.40 ± 0.06 (4)	8.26 ± 0.13 (4)
h5-ht$_{5A}$-*Sf*9	7.72 ± 0.03[c] (3)	6.85 ± 0.08[c] (7)	6.67 ± 0.14[c] (3)	7.38 ± 0.17 (3)	8.64 ± 0.06[c] (3)	8.44 ± 0.15 (3)
h5-ht$_{5A}$ + Gβ$_{1\gamma_2}$-*Sf*9	7.95 ± 0.09[c] (3)	6.94 ± 0.13[c] (3)	6.79 ± 0.04[c] (3)	7.41 ± 0.12[c] (3)	8.61 ± 0.16 (3)	8.21 ± 0.27 (3)
h5-ht$_{5A}$ + Gα$_{i/o}$ + Gβ$_1$γ$_2$-*Sf*9[b]	8.26 ± 0.12 (3)	7.37 ± 0.03 (3)	7.21 ± 0.12 (3)	6.82 ± 0.18[c] (3)	8.08 ± 0.13[c] (3)	7.19 ± 0.01[c] (3)
h5-ht$_{5A}$ + Gα$_{i1}$ + Gβ$_1$γ$_2$-*Sf*9	8.35 ± 0.09 (3)	7.61 ± 0.28 (3)	7.20 ± 0.15 (3)	7.14 ± 0.08 (3)	8.43 ± 0.14 (3)	7.49 ± 0.29[c] (3)
h5-ht$_{5A}$ + Gα$_{i2}$ + Gβ$_1$γ$_2$-*Sf*9	8.23 ± 0.10 (3)	7.35 ± 0.32 (3)	7.16 ± 0.08 (3)	7.29 ± 0.06 (3)	8.44 ± 0.10 (3)	7.72 ± 0.18[c] (3)
h5-ht$_{5A}$ + Gα$_{i3}$ + Gβ$_1$γ$_2$-*Sf*9	8.24 ± 0.11 (3)	7.28 ± 0.21 (3)	7.19 ± 0.15 (3)	7.13 ± 0.09 (3)	8.45 ± 0.03 (3)	7.49 ± 0.19[c] (3)
h5-ht$_{5A}$ + Gα$_o$ + Gβ$_1$γ$_2$-*Sf*9	8.21 ± 0.12 (3)	7.28 ± 0.17 (3)	6.99 ± 0.09 (3)	7.18 ± 0.04 (3)	8.17 ± 0.09[c] (3)	7.83 ± 0.11[c] (3)
h5-ht$_{5A}$ + Gα$_z$ + Gβ$_1$γ$_2$-*Sf*9	8.01 ± 0.07[c] (3)	7.14 ± 0.18[c] (3)	6.81 ± 0.05[c] (3)	7.29 ± 0.19 (3)	8.37 ± 0.07 (3)	8.07 ± 0.11 (3)
h5-ht$_{5A}$ + Gα$_{s-s}$ + Gβ$_1$γ$_2$-*Sf*9	8.09 ± 0.03[c] (3)	7.10 ± 0.04[c] (3)	6.88 ± 0.04[c] (3)	7.02 ± 0.50 (3)	8.51 ± 0.08 (3)	8.37 ± 0.07 (3)

[a] Data for h5-ht$_{5A}$-HEK 293 were taken from Francken et al., 1998.
[b] Gα$_{i/o}$ represents the combination of Gα$_{i1}$, Gα$_{i2}$, Gα$_{i3}$, and Gα$_o$ subunits.
[c] pIC$_{50}$ value that is significantly (Student's *t* test, $P < .05$) different from the corresponding pIC$_{50}$ value for h5-ht$_{5A}$-HEK 293.

activity in transfected HEK 293 cells (Francken et al., 1998; Hurley et al., 1998). High-affinity agonist binding and agonist-stimulated [^{35}S]GTPγS binding to h5-ht$_{5A}$-HEK 293 membranes were found to be pertussis toxin-sensitive (Francken et al., 1998), indicating the involvement of G_i/G_o proteins. To provide further insight in its signaling properties, we coexpressed the h5-ht$_{5A}$ receptor in *Sf*9 insect cells with a series of 11 mammalian G proteins, from each of the four Gα families. Using radioligand and [^{35}S]GTPγS binding assays, we demonstrated selective coupling of the h5-ht$_{5A}$ receptor to coexpressed G_i and G_o proteins and the absence of coupling to $G_z/G_s/G_q/G_{11}/G_{16}/G_{12}$ and G_{13} proteins. Hence, the h5-HT$_{5A}$ receptor does not show promiscuous coupling to various G protein families. Although no clear coupling pref-

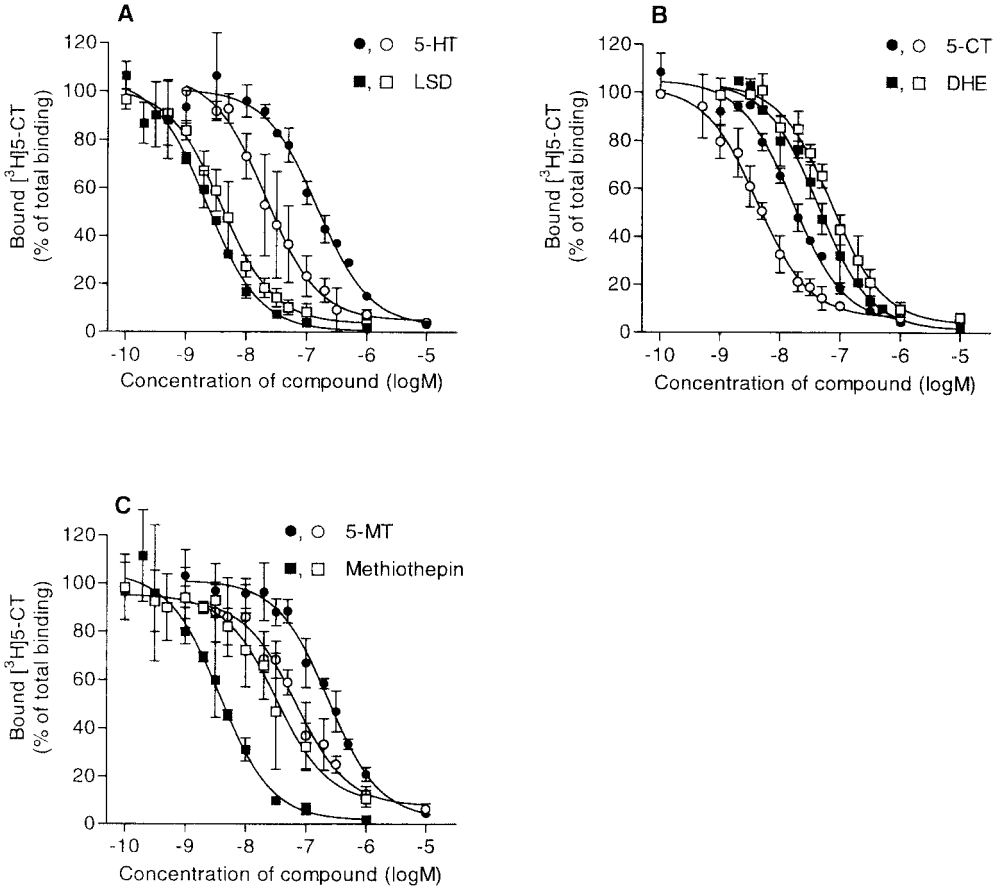

Fig. 3. Inhibition of [^{3}H]5-CT (2 nM) binding to membranes of baculovirus-infected *Sf*9 insect cells expressing h5-ht$_{5A}$ receptors alone (●, ■) or together with $G\alpha_{i1}$ and $G\beta_1\gamma_2$ subunits (○, □). A, 5-HT (●, ○) and LSD (■, □). B, 5-CT (●, ○) and DHE (■, □). C, 5-MT (●, ○) and methiothepin (■, □). Depicted points are mean ± S.D. values of three to seven independent experiments. Mean values of pIC_{50} values derived from individual curves are given in Table 2. Baculovirus infection of *Sf*9 cells and radioligand binding studies were performed as described in *Experimental Procedures*.

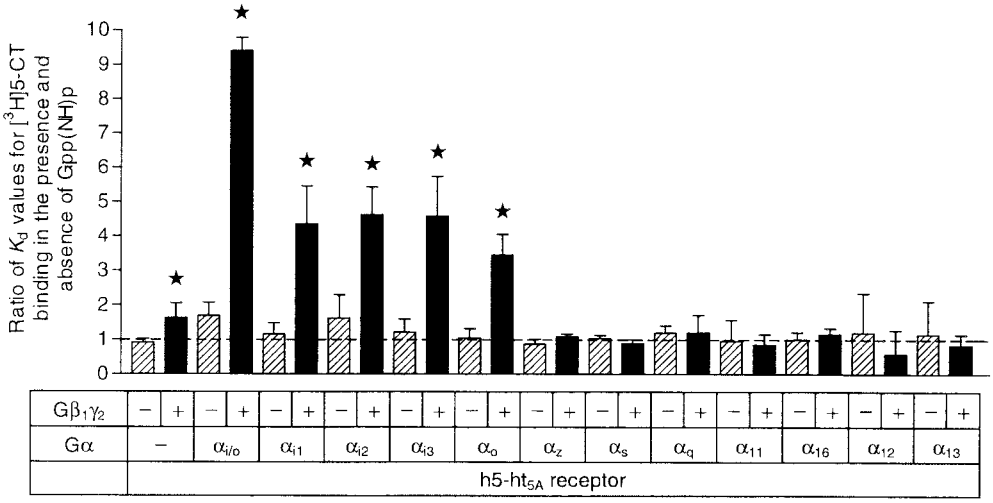

Fig. 4. Gpp(NH)p sensitivity of [^{3}H]5-CT binding to membranes of *Sf*9 cells coexpressing h5-ht$_{5A}$ receptors and diverse G protein subunits. The data represent the mean ratios (±S.D.) of the K_d values for [^{3}H]5-CT in the presence of 100 μM Gpp(NH)p versus the K_d values for [^{3}H]5-CT in the absence of Gpp(NH)p. Concentration-binding experiments were performed in parallel in the absence and presence of 100 μM Gpp(NH)p as described in *Experimental Procedures*. B_{max} and K_d values were derived for each individual experiment, and mean values are summarized in Table 1. For each individual experiment, the ratio was calculated of the K_d value in the presence versus that in the absence of 100 μM Gpp(NH)p. Comparisons were made using the paired two-tailed Student's *t* test. *Significant (Student's *t* test, $P < .05$) difference in K_d value for [^{3}H]5-CT in the presence versus in the absence of 100 μM Gpp(NH)p.

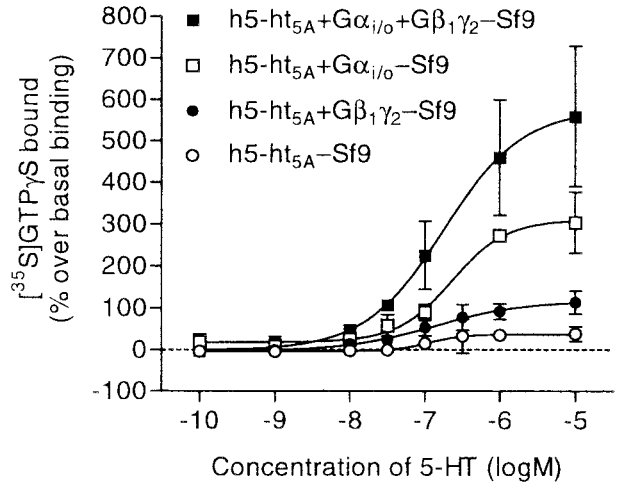

Fig. 5. 5-HT-stimulated binding of [^{35}S]GTPγS to membranes of baculovirus-infected *Sf*9 cells expressing h5-ht$_{5A}$ receptors alone or in combination with G protein subunits. ○, h5-ht$_{5A}$ receptors expressed alone. ●, h5-ht$_{5A}$ receptors coexpressed with the Gβ$_1$γ$_2$ complex. □, h5-ht$_{5A}$ receptors coexpressed with the mixture of Gα$_{i1}$, Gα$_{i2}$, Gα$_{i3}$, and Gα$_o$ subunits. ■, h5-ht$_{5A}$ receptors coexpressed with the Gβ$_1$γ$_2$ complex and the mixture of Gα$_{i1}$, Gα$_{i2}$, Gα$_{i3}$, and Gα$_o$ subunits. Membranes were preincubated with compound for 30 min at 30°C and incubated with 0.2 nM [^{35}S]GTPγS for an additional 30 min at 30°C. Basal [^{35}S]GTPγS binding was measured in the absence of 5-HT, and the percentage of stimulation was calculated as defined in *Experimental Procedure*. Depicted points are mean ± S.D. values from two to five independent experiments, each performed in duplicate. Mean pEC$_{50}$ and maximum stimulation values are summarized in Table 3.

erence to either of the G$_i$/G$_o$ subtypes was evident, we have observed differences in the coupling behavior of G$_o$ versus G$_i$.

The overexpression in *Sf*9 cells of h5-ht$_{5A}$ receptors alone resulted in a predominantly uncoupled phenotype, as demonstrated by guanine nucleotide-insensitive, low-affinity agonist binding. Although not evident from the binding data, h5-ht$_{5A}$ receptors coupled to endogenous G proteins to some extent; 5-HT stimulated [^{35}S]GTPγS binding to 40% over basal. We conclude that a large excess of uncoupled receptors is present in h5-ht$_{5A}$-*Sf*9 membranes. Although the activation of G proteins by a small fraction of coupled receptors can be detected due to the sensitivity of the [^{35}S]GTPγS binding assay, the curve-fitting algorithms for the concentration-binding isotherms cannot reliably detect a high-affinity binding component of less than 10% of the B_{max} value.

When the h5-ht$_{5A}$ receptor was coexpressed with G$_{i1}$/G$_{i2}$/G$_{i3}$ or G$_o$ proteins (Gαβ$_1$γ$_2$ heterotrimers), the coupled phenotype was achieved, as evident from guanine nucleotide-sensitive, high-affinity agonist binding. In addition, the affinity of methiothepin, which was identified as an inverse agonist at h5-ht$_{5A}$-HEK 293 cells (Francken et al., 1998), decreased on coexpression of G$_i$/G$_o$ proteins. These observations are consistent with two distinct states of the h5-ht$_{5A}$ receptor, according to the two-state model (Leff, 1995). Receptors are proposed to exist in an active form (R*) that is G protein-coupled and an inactive form (R). Agonists show high affinity for R* and low affinity for R, whereas inverse agonists display the opposite behavior (Milligan et al., 1995). Our binding data suggest that h5-ht$_{5A}$ receptors expressed in

TABLE 3

Stimulation by 5-HT of [^{35}S]GTPγS (0.2 nM) binding to membranes of *Sf*9 cells coexpressing the h5-ht$_{5A}$ receptor and G protein subunits of the G$_{i/o}$, G$_s$, G$_{q/11}$, and G$_{12/13}$ family

[^{35}S]GTPγS binding studies were performed as described in *Experimental Procedures*. The maximum stimulation and pEC$_{50}$ values were derived from the curves. The results are mean ± S.D. values from *n* independent experiments.

Proteins Expressed	Maximum Stimulation	pEC$_{50}$	*n*
	%	−*logM*	
h5-ht$_{5A}$-HEK 293[a]	135 ± 3	7.0 ± 0.1	3
h5-ht$_{5A}$-*Sf*9	38 ± 16	6.9 ± 0.4	5
h5-ht$_{5A}$ + Gα$_{i/o}$-*Sf*9[b]	326 ± 65[c]	6.5 ± 0.2	2
h5-ht$_{5A}$ + Gα$_{i1}$-*Sf*9	297 ± 120[c]	6.6 ± 0.1	3
h5-ht$_{5A}$ + Gα$_{i2}$-*Sf*9	340 ± 131[c]	6.6 ± 0.2	4
h5-ht$_{5A}$ + Gα$_{i3}$-*Sf*9	260 ± 55[c]	6.9 ± 0.1	4
h5-ht$_{5A}$ + Gα$_o$-*Sf*9	263 ± 60[c]	7.0 ± 0.1	4
h5-ht$_{5A}$ + Gα$_z$-*Sf*9	20 ± 3	6.8 ± 0.6	3
h5-ht$_{5A}$ + Gα$_s$-*Sf*9	9 ± 3	6.6 ± 0.0	2
h5-ht$_{5A}$ + Gα$_q$-*Sf*9	25 ± 13	6.5 ± 0.4	3
h5-ht$_{5A}$ + Gα$_{11}$-*Sf*9	54 ± 9	6.9 ± 0.2	3
h5-ht$_{5A}$ + Gα$_{16}$-*Sf*9	50 ± 11	6.6 ± 0.3	3
h5-ht$_{5A}$ + Gα$_{12}$-*Sf*9	—[e]	—[e]	2
h5-ht$_{5A}$ + Gα$_{13}$-*Sf*9	—[e]	—[e]	3
h5-ht$_{5A}$ + Gβ$_1$γ$_2$-*Sf*9	113 ± 23[d]	6.9 ± 0.3	5
h5-ht$_{5A}$ + Gα$_{i/o}$ + Gβ$_1$γ$_2$-*Sf*9[b]	573 ± 184	6.6 ± 0.1	2
h5-ht$_{5A}$ + Gα$_{i1}$ + Gβ$_1$γ$_2$-*Sf*9	629 ± 153[d]	6.9 ± 0.1	7
h5-ht$_{5A}$ + Gα$_{i2}$ + Gβ$_1$γ$_2$-*Sf*9	490 ± 149	6.8 ± 0.2	7
h5-ht$_{5A}$ + Gα$_{i3}$ + Gβ$_1$γ$_2$-*Sf*9	489 ± 56[d]	7.1 ± 0.1	5
h5-ht$_{5A}$ + Gα$_o$ + Gβ$_1$γ$_2$-*Sf*9	172 ± 27	7.4 ± 0.1	4
h5-ht$_{5A}$ + Gα$_z$ + Gβ$_1$γ$_2$-*Sf*9	61 ± 12[d]	7.0 ± 0.3	3
h5-ht$_{5A}$ + Gα$_s$ + Gβ$_1$γ$_2$-*Sf*9	36 ± 9[d]	6.6 ± 0.1	3
h5-ht$_{5A}$ + Gα$_q$ + Gβ$_1$γ$_2$-*Sf*9	135 ± 9[d]	6.8 ± 0.4	3
h5-ht$_{5A}$ + Gα$_{11}$ + Gβ$_1$γ$_2$-*Sf*9	81 ± 50	6.9 ± 0.6	4
h5-ht$_{5A}$ + Gα$_{16}$ + Gβ$_1$γ$_2$-*Sf*9	64 ± 18	6.4 ± 0.4	3
h5-ht$_{5A}$ + Gα$_{12}$ + Gβ$_1$γ$_2$-*Sf*9	—[e]	—[e]	2
h5-ht$_{5A}$ + Gα$_{13}$ + Gβ$_1$γ$_2$-*Sf*9	—[e]	—[e]	3

[a] Data for h5-ht$_{5A}$-HEK 293 were taken from Francken et al., 1998.
[b] Gα$_{i/o}$ represents the combination of Gα$_{i1}$, Gα$_{i2}$, Gα$_{i3}$, and Gα$_o$ subunits.
[c] Maximum stimulation value for h5-ht$_{5A}$ +Gα-*Sf*9 that is significantly (Student's *t* test, $P < .05$) higher than that for h5-ht$_{5A}$-*Sf*9.
[d] Maximum stimulation value for h5-ht$_{5A}$(+Gα) + Gβ$_1$γ$_2$-*Sf*9 that is significantly (Student's *t* test, $P < .05$) higher than that for h5-ht$_{5A}$(+Gα)-*Sf*9.
[e] —, no 5-HT-induced stimulation of [^{35}S]GTPγS binding was observed for coexpressions with Gα$_{12}$ or Gα$_{13}$ subunits.

*Sf*9 cells convert to the active, high agonist affinity state (R*) through interaction with coexpressed G_i/G_o proteins. Remarkably, the affinities of DHE and LSD, which were identified as partial agonists at h5-ht_{5A}-HEK 293 cells, decreased on coexpression of G_i and/or G_o proteins. This observation might indicate that the two-state model of agonist action is not generally applicable to partial agonists.

Evidence for h5-ht_{5A} receptor-mediated G_i/G_o protein activation was obtained using [^{35}S]GTPγS assays. The maximum level of 5-HT-stimulated [^{35}S]GTPγS binding to coexpressed G_o proteins was similar to that found for h5-ht_{5A}-HEK 293 cells, whereas $G_{i1}/G_{i2}/G_{i3}$ and the mixture of $G_{i/o}$ proteins were stimulated by 5-HT with approximately 4-fold higher efficacy. The lower level of 5-HT-mediated stimulation of G_o, compared with G_i, might be explained by the 2.6-fold higher basal [^{35}S]GTPγS binding that was found for coexpressed G_o. This high agonist-independent [^{35}S]GTPγS binding most probably originates from a larger number of G_o proteins in the *Sf*9 membranes compared with G_i, as $G\alpha_o$ appeared more abundant than the various $G\alpha_i$ subunits in immunoblot analysis. Alternatively, the h5-ht_{5A} receptor may exhibit stronger constitutive activation of G_o, compared with G_i. High agonist-independent binding complicates the detection of agonist-induced increases in [^{35}S]GTPγS binding (Wieland and Jakobs, 1994). It could be that the assay conditions (e.g., buffer composition and incubation temperature) optimal for G_o activation differ from the applied conditions, such that the actual maximum stimulation of G_o by 5-HT might well be higher than reported.

Coexpression of the h5-ht_{5A} receptor with one of the other G proteins tested ($G_z/G_s/G_q/G_{11}/G_{16}/G_{12}$ or G_{13}) had no effect on agonist binding, and no or only minor 5-HT-induced activation of these G proteins could be detected. The expression of the different heterologous Gα proteins in the *Sf*9 membranes was confirmed using immunoblotting. All Gα proteins were highly expressed, and only $G\alpha_{12}$ showed weak immunoreactivity. Hence, poor subunit expression is not the reason for the absence of effects for the various G proteins, except perhaps for $G\alpha_{12}$. It should be noted that in the [^{35}S]GTPγS studies, the assay conditions were not optimized

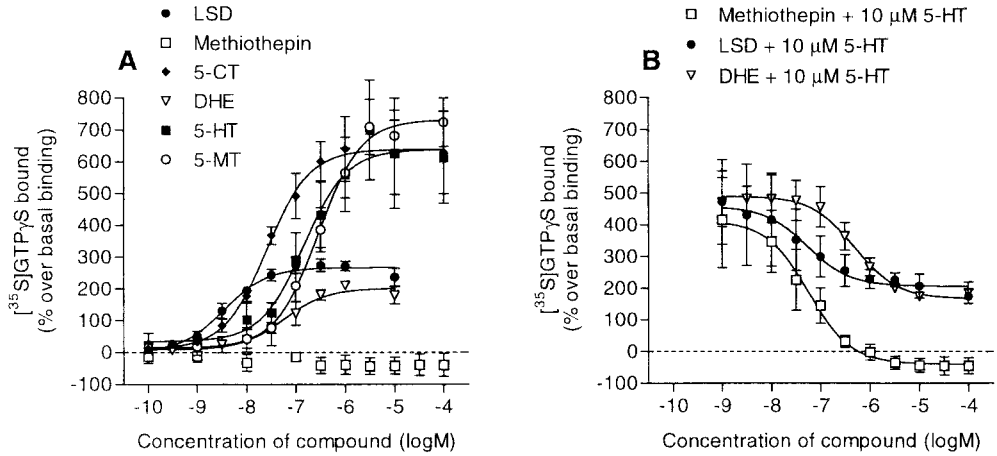

Fig. 6. [^{35}S]GTPγS binding to membranes of baculovirus-infected *Sf*9 cells coexpressing h5-ht_{5A} receptors with the $G\beta_1\gamma_2$ complex and $G\alpha_{i1}$ subunits. A, stimulation of [^{35}S]GTPγS binding by 5-HT receptor agonists. B, antagonism of 5-HT (10 μM)-stimulated [^{35}S]GTPγS binding by 5-HT receptor ligands. Membranes were preincubated with 5-HT for 30 min at 30°C and incubated with 0.2 nM [^{35}S]GTPγS for an additional 30 min at 30°C. Basal [^{35}S]GTPγS binding was measured in the absence of compounds, and the percentage of stimulation was calculated as defined in *Experimental Procedures*. Depicted points are mean ± S.D. values from two to seven independent experiments, each performed in duplicate. Mean pEC_{50}, E_{max}, pIC_{50}-corr, and I_{max} values are summarized in Table 4.

TABLE 4

Effect of various 5-HT receptor ligands on [^{35}S]GTPγS (0.2 nM) binding to membranes of *Sf*9 cells coexpressing the h5-ht_{5A} receptor and G protein subunits of the $G_{i/o}$ family

[^{35}S]GTPγS binding studies were performed on membranes as described in *Experimental Procedures*. pEC_{50} and pIC_{50} values were derived from the curves. E_{max} values and I_{max} values were calculated, and pIC_{50} values were corrected into pIC_{50}-corr values as described under *Experimental Procedures*. The results are mean ± S.D. values from *n* independent experiments.

Compound	*Sf*9 cells Expressing the h5-ht_{5A} Receptor+											
	$G\alpha_{i1} + G\beta_1\gamma_2$			$G\alpha_{i2} + G\beta_1\gamma_2$			$G\alpha_{i3} + G\beta_1\gamma_2$			$G\alpha_o + G\beta_1\gamma_2$		
	pEC_{50}	E_{max}	*n*	pEC_{50}	E_{max}	*n*	pEC_{50}	E_{max}	*n*	pEC_{50}	E_{max}	*n*
	−logM	%		*−logM*	%		*−logM*	%		*−logM*	%	
5-CT	7.6 ± 0.2	98 ± 12	3	7.6 ± 0.2	91 ± 17	3	7.9 ± 0.1	98 ± 16	3	8.0 ± 0.0	98 ± 1	2
5-MT	6.5 ± 0.1	98 ± 17	3	6.4 ± 0.1	91 ± 17	3	6.9 ± 0.2	94 ± 12	3	7.2 ± 0.0	88 ± 4	2
DHE	7.3 ± 0.5	46 ± 7	3	7.0 ± 0.2	44 ± 8	3	7.0 ± 0.1	46 ± 19	3	7.4 ± 0.3	82 ± 9	3
LSD	8.5 ± 0.2	64 ± 23	3	8.4 ± 0.2	55 ± 14	3	8.2 ± 0.2	58 ± 1	3	7.9 ± 0.9	93 ± 4	3
	pIC_{50}-corr	I_{max}	*n*	pIC_{50}-corr	I_{max}	*n*	pIC_{50}-corr	I_{max}	*n*	pIC_{50}-corr	I_{max}	*n*
DHE	8.0 ± 0.1	64 ± 7	3	8.0 ± 0.1	65 ± 7	3	8.2 ± 0.1	62 ± 8	3	8.8 ± 0.1	13 ± 7	2
LSD	9.0 ± 0.2	52 ± 13	3	9.0 ± 0.2	56 ± 11	3	9.1 ± 0.4	44 ± 3	3	9.1 ± 0.5	17 ± 13	3
Methiothepin	8.9 ± 0.5	111 ± 6	3	8.9 ± 0.5	110 ± 3	3	9.2 ± 0.0	110 ± 4	3	9.1 ± 0.2	123 ± 2	3

for each individual G protein type. Under the applied conditions, which were optimized for [^{35}S]GTPγS binding to h5-ht$_{5A}$-HEK 293 membranes, activation of some G protein types may therefore be suboptimal. As the need to optimize assay conditions for individual G proteins has been reported previously (Wieland and Jacobs, 1994), it would be rash to conclude the absolute absence of h5-ht$_{5A}$ receptor coupling to G$_z$/G$_s$/G$_q$/G$_{11}$/G$_{16}$/G$_{12}$ or G$_{13}$ proteins based exclusively on the absence of increases in [^{35}S]GTPγS binding. The lack of receptor interaction with these G proteins is only suggested by the fact that coexpression of these G proteins did not induce guanine nucleotide-sensitive, high-affinity agonist binding to the h5-ht$_{5A}$ receptor. It appears that h5-ht$_{5A}$ receptors selectively couple to G$_i$/G$_o$ proteins, which is in agreement with the finding that pertussis toxin pretreatment completely abolished high-affinity agonist binding and 5-HT-stimulated [^{35}S]GTPγS binding to h5-ht$_{5A}$-HEK 293 membranes (Francken et al., 1998).

The Gβγ complex has already been shown to be required for optimal receptor-G protein interaction (Fung, 1983; Butkerait et al., 1995). We have used the Gβ$_1$γ$_2$ dimer to enhance G protein coupling to the h5-ht$_{5A}$ receptor, because this dimer was reported to interact with members of the four Gα families (Barr et al., 1997). However, the subunit composition of Gβγ affects receptor-G protein coupling specificity (Kisselev and Gautam, 1993; Kleuss et al., 1993; Richardson and Robishaw, 1999), such that other Gβγ subunit compositions may yield different receptor coupling profiles. Therefore, we also investigated h5-ht$_{5A}$ receptor-G protein coupling in the absence of the mammalian Gβ$_1$γ$_2$ complex. The interaction of receptor with Gα$_{i1}$, Gα$_{i2}$, and Gα$_{i3}$ could still be detected in agonist binding and [^{35}S]GTPγS assays, but it was indeed less effective than that in the presence of Gβ$_1$γ$_2$. Remarkably, coexpression with Gα$_o$ did not induce high-affinity agonist binding in the absence of Gβ$_1$γ$_2$. Previously, Jockers et al. (1994) found similar results for adenosine A$_1$ receptors expressed in *Escherichia coli*; reconstitution of high-affinity agonist binding by purified G proteins was poor in the absence of Gβγ for G$_o$, but not for G$_i$, whereas in the presence of Gβγ, their maximum responses were similar. Despite the lack of effect on agonist affinity of Gα$_o$, the activation of h5-ht$_{5A}$ receptors produced a maximum stimulation of [^{35}S]GTPγS binding similar to Gα$_i$ subunits. Coexpression of h5-ht$_{5A}$ receptors and either Gα$_z$/Gα$_s$/Gα$_q$/Gα$_{11}$/Gα$_{16}$/Gα$_{12}$ or Gα$_{13}$ without Gβ$_1$γ$_2$ did not result in the coupled phenotype, as expected from the lack of effect when Gαβ$_1$γ$_2$ heterotrimers were expressed. We conclude that the Gβ$_1$γ$_2$ complex greatly facilitates coupling of G$_{i/o}$ proteins to the h5-ht$_{5A}$ receptor when coexpressed in *Sf*9 cells.

Coexpression of h5-ht$_{5A}$ receptors and Gβ$_1$γ$_2$ without mammalian Gα subunits revealed that Gβ$_1$γ$_2$ enhances interaction of heterologous receptor with insect G proteins. Similar results were reported for the serotonin 5-HT$_{1A}$ and the dopamine D$_{2S}$ receptor (Butkerait et al., 1995; Boundy et al., 1996). Considering this finding, one should note that an improved interaction of recombinant receptors with endogenous G proteins due to coexpressed Gβ$_1$γ$_2$ subunits may confuse the interpretation of receptor-G protein interaction specificity. Regardless, it is clear that the overexpression of specifically interacting G proteins should yield effects that exceed these observed for the appropriate controls.

For some receptors that couple to pertussis toxin-sensitive G proteins, preferential interaction with one of the G$_i$/G$_o$ subtypes has been demonstrated (Senogles et al., 1990; Parker et al., 1991; Rubinstein et al., 1991; Grünewald et al., 1996; Clawges et al., 1997; Lorenzen et al., 1998). Our data indicate that the heterotrimeric G$_{i1}$, G$_{i2}$, G$_{i3}$, or G$_o$ proteins interacted equally well with the h5-ht$_{5A}$ receptor to induce its high-affinity conformation, and no significant differences in the affinities of the tested compounds were observed. However, in contrast to Gα$_i$, Gα$_o$ did not induce high-affinity agonist binding in the absence of Gβ$_1$γ$_2$, suggesting diminished receptor interaction. Furthermore, some striking differences between G$_o$ and G$_i$ proteins appeared from the [^{35}S]GTPγS experiments. Maximum stimulation of [^{35}S]GTPγS binding by 5-HT was significantly lower at G$_o$ than at G$_i$, possibly due to the high agonist-independent [^{35}S]GTPγS binding to G$_o$. In addition, the relative efficacies of DHE and LSD were dependent on the G protein type expressed. Both compounds were full agonists at the h5-ht$_{5A}$ receptor when coexpressed with G$_o$, whereas coexpression with G$_i$ proteins resulted in partial agonistic behavior, which was also found at the h5-HT$_{5A}$-HEK 293 membranes (Francken et al., 1998). These data might be explained by a difference in receptor/G protein stoichiometry, which can influence both agonist potency and efficacy (Hermans et al., 1999). Alternatively, the efficacy of compounds may be determined by the type of G protein interacting with the receptor. In this respect, Yang and Lanier (1999) have reported that recombinant expression of Gα$_o$, but not Gα$_{i1}$, increased the relative efficacy of clonidine in NIH-3T3 cells cotransfected with α$_2$-adrenergic receptor and Gα subunit, an effect that was not an issue of G protein or receptor levels. Although we cannot exclude that differences in h5-ht$_{5A}$ receptor-to-G protein ratio cause the distinct behavior of G$_o$ and G$_i$ proteins, it is tempting to speculate that structural differences exist in their interaction with the h5-HT$_{5A}$ receptor. However, differences in the nucleotide binding properties of the G protein types themselves should also be taken into account; as such, G$_o$ may be easier to activate by receptors than G$_i$.

In summary, the h5-HT$_{5A}$ receptor selectively coupled to mammalian G$_{i1}$/G$_{i2}$/G$_{i3}$ and G$_o$ but not to G$_z$/G$_s$/G$_{q/11/16}$ or G$_{12/13}$ proteins, when coexpressed in *Sf*9 insect cells. Although G$_o$ displayed different receptor coupling characteristics than G$_i$ proteins, no clear coupling preference was evident.

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References

Barr AJ, Brass LF and Manning DR (1997) Reconstitution of receptors and GTP-binding regulatory proteins (G proteins) in *Sf*9 cells: Direct evaluation of selectivity in receptor-G protein coupling. *J Biol Chem* **272:**2223–2229.

Boundy VA, Lu L and Molinoff PB (1996) Differential coupling of rat D2 dopamine receptor isoforms expressed in *Spodoptera frugiperda* insect cells. *J Pharmacol Exp Ther* **276:**784–794.

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248–254.

Butkerait P, Zheng Y, Hallak H, Graham TE, Miller HA, Burris KD, Molinoff PB and Manning DR (1995) Expression of the human 5-hydroxytryptamine$_{1A}$ receptor in *Sf*9 cells. Reconstitution of a coupled phenotype by co-expression of mammalian G protein subunits. *J Biol Chem* **270:**18691–18699.

Carson MJ, Thomas EA, Danielson PE and Sutcliffe JG (1996) The 5-HT$_{5A}$ serotonin receptor is expressed predominantly by astrocytes in which it inhibits cAMP accumulation: A mechanism for neuronal suppression of reactive astrocytes. *Glia* **17:**317–326.

Clawges HM, Depree KM, Parker EM and Graber SG (1997) Human 5-HT$_1$ receptor subtypes exhibit distinct G protein coupling behaviors in membranes from *Sf*9 cells. *Biochemistry* **36:**12930–12938.

Erlander MG, Lovenberg TW, Baron BM, de Lecea L, Danielson PE, Racke M, Slone AL, Siegel BW, Foye PE, Cannon K, Burns JE and Sutcliffe JG (1993) Two members of a distinct subfamily of 5-hydroxytryptamine receptors differentially expressed in rat brain. *Proc Natl Acad Sci USA* **90:**3452–3456.

Francken BJB, Jurzak M, Vanhauwe JFM, Luyten WHML and Leysen JE (1998) The human 5-HT$_{5A}$ receptor couples to G_i/G_o proteins and inhibits adenylate cyclase in HEK 293 cells. *Eur J Pharmacol* **361:**299–309.

Fung BK (1983) Characterization of transducin from bovine retinal rod outer segments. I. Separation and reconstitution of the subunits. *J Biol Chem* **258:**10495–10502.

Graber SG, Figler RA and Garrison JC (1992) Expression and purification of functional G protein alpha subunits using a baculovirus expression system. *J Biol Chem* **267:**1271–1278.

Grailhe R, Waeber C, Dulawa SC, Hornung JP, Zhuang X, Brunner D, Geyer MA and Hen R (1999) Increased exploratory activity and altered response to LSD in mice lacking the 5-HT$_{5A}$ receptor. *Neuron* **22:**581–591.

Grünewald S, Reiländer H and Michel H (1996) *In vivo* reconstitution of dopamine D_{2S} receptor-mediated G protein activation in baculovirus-infected insect cells: Preferred coupling to G_{i1} versus G_{i2}. *Biochemistry* **35:**15162–15173.

Hepler JR, Kozasa T, Smrcka AV, Simon MI, Rhee SG, Sternweis PC and Gilman AG (1993) Purification from *Sf*9 cells and characterization of recombinant $G_{q\alpha}$ and $G_{11\alpha}$: Activation of purified phospholipase C isozymes by G_α subunits. *J Biol Chem* **268:**14367–14375.

Hermans E, Challiss RAJ and Nahorski SR (1999) Effects of varying the expression level of recombinant human mGlu1α receptors on the pharmacological properties of agonists and antagonists. *Br J Pharmacol* **126:**873–882.

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

Hurley PT, McMahon RA, Fanning P, O'Boyle KM, Rogers M and Martin F (1998) Functional coupling of a recombinant human 5-HT$_{5A}$ receptor to G-proteins in HEK-293 cells. *Br J Pharmacol* **124:**1238–1244.

Jockers R, Linder ME, Hohenegger M, Nanoff C, Bertin B, Strosberg AD, Marullo S and Freissmuth M (1994) Species difference in the G protein selectivity of the human and bovine A_1-adenosine receptor. *J Biol Chem* **269:**32077–32084.

Kisselev O and Gautam N (1993) Specific interaction with rhodopsin is dependent on the γ subunit type in a G protein. *J Biol Chem* **268:**24519–24522.

Kleuss C, Scherubl H, Hescheler J, Schultz G and Wittig B (1993) Selectivity in signal transduction determined by γ subunits of heterotrimeric G proteins. *Science (Wash DC)* **259:**832–834.

Kozasa T, Hepler JR, Smrcka AV, Simon MI, Rhee SG, Sternweis PC and Gilman AG (1993) Purification and characterization of recombinant $G_{16}\alpha$ from *Sf*9 cells: Activation of purified phospholipase C isozymes by G-protein α subunits. *Proc Natl Acad Sci USA* **90:**9176–9180.

Leff P (1995) The two-state model of receptor activation. *Trends Pharmacol Sci* **16:**89–97.

Linder ME, Middleton P, Hepler JR, Taussig R, Gilman AG and Mumby SM (1993) Lipid modifications of G proteins: α Subunits are palmitoylated. *Proc Natl Acad Sci USA* **90:**3675–3679.

Lorenzen A, Lang H and Schwabe U (1998) Activation of various subtypes of the G-protein α subunits by partial agonists of the adenosine A_1 receptor. *Biochem Pharmacol* **56:**1287–1293.

Matthes H, Boschert U, Amlaiky N, Grailhe R, Plassat J-L, Muscatelli F, Mattei M-G and Hen R (1993) Mouse 5-hydroxytryptamine$_{5A}$ and 5-hydroxytryptamine$_{5B}$ receptors define a new family of serotonin receptors: Cloning, functional expression, and chromosomal localization. *Mol Pharmacol* **43:**313–319.

Milligan G, Bond RA and Lee M (1995) Inverse agonism: Pharmacological curiosity or potential therapeutic strategy? *Trends Pharmacol Sci* **16:**10–13

Nakamura F, Kato M, Kameyama K, Nukada T, Haga T, Kato H, Takenawa T and Kikkawa U (1995) Characterization of G_q family G proteins $G_{L1}\alpha$ ($G_{14}\alpha$), $G_{L2}\alpha$ ($G_{11}\alpha$), and $G_q\alpha$ expressed in the baculovirus-insect cell system. *J Biol Chem* **270:**6246–6253.

Oestreicher EG and Pinto GF (1987) A microcomputer program for fitting enzyme inhibition equations. *Comput Biol Med* **17:**53–68.

Ohtaki T, Ogi K, Masuda Y, Mitsuoka K, Fujiyoshi Y, Kitada C, Sawada H, Onda H and Fujino M (1998) Expression, purification, and reconstitution of receptor for pituitary adenylate cyclase-activating polypeptide: Large-scale purification of a functionally active G protein-coupled receptor produced in *Sf*9 insect cells. *J Biol Chem* **273:**15464–15473.

O'Reilly DR, Miller LK and Luckow VA (1992) *Baculovirus Expression Vectors: A Laboratory Manual*. W. H. Freeman, New York.

Parker EM, Kameyama K, Higashijima T and Ross EM (1991) Reconstitutively active G protein-coupled receptors purified from baculovirus-infected insect cells. *J Biol Chem* **266:**519–527.

Plassat J-L, Boschert U, Amlaiky N and Hen R (1992) The mouse 5-HT$_5$ receptor reveals a remarkable heterogeneity within the 5-HT$_{1D}$ receptor family. *EMBO J* **11:**4779–4786.

Rees S, den Daas I, Foord S, Goodson S, Bull D, Kilpatrick G and Lee M (1994) Cloning and characterisation of the human 5-HT$_{5A}$ serotonin receptor. *FEBS Lett* **355:**242–246.

Richardson M and Robishaw JD (1999) The α_{2A}-adrenergic receptor discriminates between G_i heterotrimers of different $\beta\gamma$ subunit composition in *Sf*9 insect cell membranes. *J Biol Chem* **274:**13525–13533.

Rubinstein RC, Linder ME and Ross EM (1991) Selectivity of the β-adrenergic receptor among G_s, G_i's, and G_o: Assay using recombinant α subunits in reconstituted phospholipids. *Biochemistry* **30:**10769–10777.

Saudou F and Hen R (1994) 5-Hydroxytryptamine receptor subtypes in vertebrates and invertebrates. *Neurochem Int* **25:**503–532.

Senogles SE, Spiegel AM, Padrell E, Iyengar R and Caron MG (1990) Specificity of receptor-G protein interactions: Discrimination of G_i subtypes by the D_2 dopamine receptor in a reconstituted system. *J Biol Chem* **265:**4507–4514.

Wieland T and Jakobs KH (1994) Measurement of receptor-stimulated guanosine 5′-O-(γ-thio)triphosphate binding by G proteins. *Methods Enzymol* **237:**3–13.

Yang Q and Lanier SM (1999) Influence of G protein type on agonist efficacy. *Mol Pharmacol* **56:**651–656.

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