Membranes prepared after infection of Sf9 cells with recombinant baculovirus containing the rat 5HT_{2c} receptor DNA, but not after infection with wild-type virus, expressed high affinity binding sites for ^{125}I-lysergic acid diethylamide and ^{3}Hmesulergine. The receptor site density reached an optimum of 50–70 pmol/mg membrane protein at 60 h postinfection. Extraction of peripheral membrane proteins from the postnuclear membrane fraction with 6 M urea depleted GTPγS-binding 4-fold without decreasing 5HT_{2c} receptor binding activity. Urea-extracted Sf9 membranes expressing the 5HT_{2c} receptor catalyzed the activation of squid retinal αq but not bovine retinal α or bovine α/γ. Productive interaction of 5HT_{2c} receptors with squid αq was enhanced by the addition of βγ dimers prepared from either bovine brain or bovine rod outer segment discs. While the addition of serotonin increased 5HT_{2c} receptor-catalyzed GTPγS-binding to αq, the unoccupied receptor was also catalytically active. The 5HT_{2c} receptor antagonists, mesulergine, mianserin, and ketanserin competitively inhibited 5HT activation of the receptor with predicted rank-order affinities; and mianserin and ketanserin markedly inhibited basal 5HT_{2c} receptor activity. Interestingly, this “inverse agonist” efficacy did not correlate with antagonist affinity for the 5HT_{2c} receptor. Baculoviral expression of the 5HT_{2c} receptor and urea extraction of postnuclear Sf9 cell membranes have provided a high density of in situ, uncoupled, G-protein-linked receptor useful for reconstitution with purified G-protein subunits. This has allowed for independent manipulation of receptor and G-protein chemical concentrations and has revealed that a G-protein-linked receptor can possess a significant basal catalytic activity and that antagonist compounds can act as inverse agonists of this basal activity at the level of receptor activation of G-proteins.

The 5HT_{2c} receptor belongs to a recently reclassified subfamily of 5-hydroxytryptamine receptors (1) and to the larger superfamily of rhodopsin-homologous, seven transmembrane domain receptors (2). Rhodopsin-homologous receptors transmit extracellular signals across biological membranes by activating specific signal transducing GTP-binding proteins (heterotrimeric G-proteins) that in turn regulate a variety of intracellular effector pathways (3–5). Agonist stimulation of the 5HT_{2c} subfamily of serotonin receptors initiates intracellular phosphoinositide (PI) hydrolysis and a subsequent rise in intracellular calcium (6, 7). The 5HT_{2c} receptor is distinguished from 5HT_{2a} and 5HT_{2b} by its ligand affinities and tissue distribution. The 5HT_{2c} receptor is expressed in neurons of the central nervous system, with especially high levels of expression in the choroid plexus (8). The neurotransmitter, serotonin, has been extensively studied and has been implicated in mental diseases ranging from affective and mood disorders to autism and dementia (9). As we more fully understand the physiological diversity exhibited by the large family of serotonin receptors (10), we will gain further insights into the neuropharmacology of serotonin.

Members of the 5HT_{2} receptor subfamily, as do other members of the extensive gene family of rhodopsin homologous receptors, signal through heterotrimeric G-proteins. Members of this receptor family, when stimulated, act to catalyze the exchange of GTP for tightly bound GDP on the G-protein α subunits, which in turn activate intracellular effectors such as adenyl cyclase or phospholipase C-β subtypes (PLC-βs). The α subunits are subclassified into four groups based upon sequence homology (11, 12). At least four α gene products have been found to stimulate phosphoinositide hydrolysis by activating PLC-βs (13); these are designated as the α_{1} subfamily (14, 15). Gβγ subunits have also been noted to activate PLC-βs (16, 17).

Because the 5HT_{2} subfamily of receptors stimulate PI hydrolysis it has been assumed that agonist stimulation of 5HT_{2c} receptors leads to GDP-GTP exchange on α_{q} which in turn activates a PLC-β isozyme(s). However, no reports have directly examined the activation of G_{q} proteins by 5HT_{2c} receptors. The in vitro studies of activation of G-proteins by receptors have proven difficult. In general, difficulties relate to purification of the quantities of proteins necessary to study the molecular interactions between a single receptor and distinct G-proteins (18, 19). Thus, although previous work has led to the inference that 5HT_{2c} activates α_{q}, the coupling of 5HT_{2c} and α_{q}, like that of many receptors with purified G-protein subunits, has not been studied directly.

With the identification of over 100 cDNAs encoding seven transmembrane domain receptors (exclusive of olfaction) and the molecular cloning of cDNAs encoding multiple gene products for each of the G-protein subunit families, a central issue in cell regulation is how these protein families contribute to the diversity of cellular responses while conserving the specificity of each response. One level of specificity is likely encoded in the...
thawed and washed by dilution with Solution C (20 mM Tris/HCl, pH 8, 1 mM EDTA, 3 mM MgCl₂, 1 mM DTT) with 5–10 plaque-forming units/cell, and collection at 20,000 rpm in a Beckman JA20 rotor. The washed membranes were then extracted at 1 mg/ml protein with Solution C containing 1% cholate by stirring at 4 °C for 30 min. The extracted membranes were sedimented at 50,000 rpm for 1 h in a Beckman type 35 rotor, and the supernatant was collected. The pelleted fraction, which contains G-protein-depleted rhodopsin, was washed with dilution of Solution B, and centrifugation at 20,000 rpm for 20 min in a Beckman JA20 rotor, resuspended with Solution B, and stored at −80 °C. The cholate-extracted supernatant was treated by sequential addition of MgCl₂ to 11 mM, AlCl₃ to 100 mM, and NaF to 10 mM (AMP) and incubated at room temperature for 30 min. The AMP-treated extract was then chilled and applied to a 200-ml (2.6 × 40 cm) column of DEAE-Sephacel (Pharmacia Biotech) that had been equilibrated with 3-bed volumes of Solution B (20 mM Tris, pH 8, 1 mM EDTA, 3 mM MgCl₂, 1 mM DTT, 1% sodium cholate, 20 mM AlCl₃, and 10 mM NaF). After application of the extract, the column was washed with 1-bed volume of Solution D and then eluted with a 4-bed volume gradient from 0 to 200 mM NaCl in Solution D, collecting 100 fractions and monitoring protein concentration by absorbance at 280 nm. All chromatography was performed at 4 °C. GTP-binding activity of purified αₛ was detected by reconstitution of chromatography fractions with squid rhodopsin, as described below, and typically eluted around 80 mM NaCl. Squid rhodopsin catalyzed GTP·S binding to a protein with an apparent molecular mass of 44 kDa, as predicted for squid transducin (αₛ,G). The DEAE chromatography fractions were visualized by SDS-polyacrylamide gel electrophoresis, pooled according to purity and activity, concentrated on a 30-kDa membrane, and subjected to gel filtration on Ultragel AcA44 (IBF) using Solution E (20 mM Tris, pH 8, 1 mM EDTA, 100 mM NaCl, 0.2% cholate). Fractions were evaluated by functional reconstitution, SDS-polyacrylamide gel electrophoresis, and amido black protein measurement (30). The typical yield for 100 retinas was 2 mg of purified αₛ. Aliquots of pooled peak fractions were stored at −80 °C. When necessary, αₛ was concentrated on centricon 30 spin concentrators (Amicon) and desalted over G-50 resin using Solution E.

βγ subunits were purified from bovine cortex (31) and, along with transducin (αₛ), from bovine rod outer segment discs (20, 32, 33) using previously published protocols.

Functional Reconstitution of In Situ 5HT₂c Receptors or Squid Rhodopsin with αₛ—The receptor-catalyzed binding of GTP·S by squid αₛ was determined by modification of the method described for bovine rhodopsin-transducin (20, 21). Reactions were carried out in 12 × 75 siliconized borosilicate glass test tubes at a volume of 50 μl of Solution F (50 mM MOPS, pH 7.5, 100 mM NaCl, 1 mM EDTA, 3 mM MgSO₄, 1 mM DTT, 1% glycerol, 1 mg/ml bovine serum albumin (BSA)) containing 1 μM αₛ and 1 μM GTP·S with trivalent ATP·γ[S](GTP·S) (about 1000 dpm/μmol). Excess radioligands were initiated by addition of Solution B membranes or squid rhodopsin, incubated in a 30 °C water bath for varying times, stopped by addition of 2 ml of Solution G (20 mM Tris/HCl, pH 8, 25 mM MgCl₂, 100 mM NaCl) at 4 °C, and immediately filtered over nitrocellulose membranes on a vacuum manifold by washing four times with 2 ml of cold Solution G. Filters were dried and radioactivity quantitated by liquid scintillation in a Wallac 1219 beta counter.

Quantitation of 5HT₂c Receptor Sites—5HT₂c binding sites were quantitated by analysis of [³H]mesulergine or [¹⁴C]-LSD binding to membrane preparations. Radioligand binding assays were carried out in solution F at 30 °C identically as functional reconstitution except that reactions were filtered over GF-G glass fiber filters. [¹¹C]-LSD was quantitated on a Wallac 1470 gamma counter. [³H]Mesulergine was counted on the Wallac 1219 beta counter.

Analysis of Data and Curve Fitting—All results shown are representative of data obtained from three or more separate experiments. All curves presented were best fits to a simple exponential model for progress curves or a single site binding model for saturation isotherms using the program “Enzfitter.”

Materials—The 5HT₂c receptor cDNA was kindly provided by Beth Hoffman (LCB, NIMH). The baculovirus DNA and transfer plasmid were purchased from Clontech. SF-900 serum-free medium was from Life Technologies, Inc. [N-6-methylen-³H]Mesulergine was obtained from Amersham Corp. 2-[¹⁴C]-Iodo-lysergic acid diethylamide and [¹¹C]GTP·S were from DuPont NEN. Squid (Loligo forbesi) eyes were purified according to the characterization of the squid photo transduction biochemistry is an ongoing project in the laboratory. The reconstitution of rhodopsin activation of Gₛ and phospholipase C isolated from squid retina will be the subject of another report.
chased from Calamari, Inc., in Woods Hole, MA. The serine protease 
 inhibitor, (4-(2-aminoethyl)-benzencesulfonylfluoride, HCl) (AEBSF), 
 was purchased from Calbiochem. Cholic acid was obtained from Sigma 
 and repurified as described (34). MOPS was from Fluka. The DEAE- 
 Sephacel and Ultrogel ACA-44 chromatography resins came from Phar- 
 macia and IBF, respectively. Bovine serum albumin used as standard 
 for protein measurements was from Pierce. The hydrochloride salts of 
 mesulergine, mianserin, and ketanserin were obtained from Research 
 Biochemicals, Inc. GTPγS was from Sigma. HAWP nitrocellulose filters 
 and the vacuum manifold were from Millipore. The GF-C glass fiber 
 filters were from Whatman.

RESULTS

After co-transfection of Sf9 cells with baculoviral DNA and the 5HT2c-containing transfer plasmid, recombinant baculovi- 
 rous was plaque-purified twice. Three of three isolated plaques 
 expressed 125I-LSD-binding sites. One of these was selected, 
 amplified, and used to obtain a viral stock that was utilized in 
 all subsequent infections (29). The time course of 5HT2c recep- 
 tor expression was assessed by 125I-LSD labeling of receptor 
 sites and by activity of membranes reconstituted with 
 αq at 12-h intervals after viral infection. We observed maximal ex- 
 pression at 60 h, by both receptor site abundance and func- 
 tional activity (data not shown). Infecting with multiplicities of 
 infection of 5 and 40 plaque-forming units/cell provided an 
 equal abundance of 5HT2c receptor sites at 60 h post-infection 
 (data not shown). 5HT2c receptor expression was diminished by 
 20–40% when recombinant baculovirus-infected Sf9 cells were 
 cultured in the presence of 1 μM mianserin, consistent with 
 findings of others (35) (data not shown). Membranes prepared 
 from Sf9 cells infected with the wild-type baculovirus vector did 
 not have specific binding sites for 125I-LSD or 3Hmesulergine, 
 and they did not catalyze GTPγS-binding to αq (data not 
 shown).

Infection of a 0.5-liter suspension culture (5 × 10⁶ cells) 
 yielded approximately 20 mg of total membrane protein in the 
 postnuclear fraction. Urea extraction reduced 60–70% of the 
 membrane-associated protein. Five infections have yielded 
 specific activities of 50–70 pmol/mg total membrane protein which 
 were increased to 150–210 pmol/mg after urea extraction. Fig. 
 1 shows the results of an experiment analyzing the saturation of 
 5HT2c binding sites with 3Hmesulergine in urea-extracted 
 membranes. These data are consistent with the expression of a 
 single class of binding sites with a Kd of 5 nM for mesulergine 
 and 190 pmol binding per mg of membrane protein in this 
 preparation.

The ability of Sf9-expressed 5HT2c receptor to couple func- 
 tionally to squid photoreceptor αq was examined in the exper- 
 iment presented in Table I. Urea extraction reduced the 
 GTPγS-binding activity of the Sf9 membranes by 75%. The functional activity of the receptor to catalyze GTPγS binding by 
 added αq was retained following urea treatment, and the ratio 
 of this catalyzed binding to the endogenous binding of GTPγS 
 by the membranes was increased from 3- to 10-fold. Reconsti- 
 tution of membranes with βγ did not increase GTPγS binding to 
 endogenous sites but enhanced the binding when co-reconsti- 
 tuted with αq, and neither αq nor βγ nor their combination 
 bound GTPγS significantly in the absence of the 5HT2c receptor 
 (data not shown). This is evidence for the presence of an 
 uncoupled receptor and is also consistent with a catalytic role for 
 βγ in 5HT2c receptor-catalyzed GTPγS-binding by αq similar to 
 that observed for reconstitution of bovine rhodopsin with αq 
 (21).

5HT activated the 5HT2c receptor to increase catalysis of 
 GTPγS binding by αq, but mianserin did not. Upon examining 
 nonextracted membranes containing the 5HT2c receptor, we 
 noted that the addition of 5HT did not increase GTPγS binding 
 by the membranes. Even the addition of saturating βγ did not 
 reveal a 5HT-stimulated GTPγS binding to endogenous Sf9 
 proteins while significantly enhancing that to added squid reti- 
 nal αq. Although others have noted an αq endogenous to Sf9 
 cells (36), the rat 5HT2c receptor does not appear to couple 
 effectively to this protein. The 5HT2c receptor in urea-extracted 
 membranes also failed to catalyze GTPγS binding by bovine 
 αq or bovine brain αq/αq fractions (data not shown). We also note 
 that in both the native and urea-extracted membranes, we 
 observed a significant enhancement of GTPγS binding to αq in 
 the absence of added serotonin and mianserin inhibited this.

The experiment in Table I utilized bovine retinal βγ in order 
 to eliminate detergent additions necessary when reconstituting 
 the hydrophobic βγ structures (21). Retinal βγ samples, as seen 
 by comparing the activity without and with αq addition, contain 
 no detectable αq contamination. However, we have been unable 
 to saturate the βγ enhancement of 5HT2c activation of αq using 
 the bovine retinal protein (data not shown). Therefore we ex- 
 amined the βγ dependence of this reconstitution using bovine 
 cortical βγ preparations. In preliminary experiments we dis- 
 covered that such preparations uniformly were contaminated 
 with GTPγS binding activity which was catalyzed by the 5HT2c 
 receptor but not by bovine rhodopsin. Neither did these prep- 
 arations contain detectable pertussis toxin substrate (data not 
 shown). We were able to remove the GTPγS binding activity 
 from the bovine brain βγ preparations by additional chroma- 
 tography over fast protein liquid chromatography phenyl- 
 Sepharose, but these experiments were not uniformly success- 
 ful. As with the retinal βγ fraction, we have failed to saturate 
 the 5HT2c receptor with such brain βγ fractions, but we can 
 estimate that the Kd is about 600 nM for these preparations. 
 Parallel analyses of the saturation of bovine rhodopsin-αq activa-
 tion by these same βγ samples indicate Kd values of 20–50 
 nM (data not shown).

Our kinetic analyses of the ligand regulation of the rat 5HT2c 
 receptor included the 5HT saturation of GTPγS exchange at 
 differing concentrations of the receptor. Fig. 2 presents data 
 from one of these experiments using 4 and 12 nM receptor. 
 Consistent with a catalytic function for the receptor, the rate of 
 GTPγS binding to αq was increased at the higher receptor 

\[
\begin{align*} 
\text{Fig. 1. Analysis of 5HT}_{2c} \text{ binding site abundance. Urea-ex}
\end{align*}
\]
In Situ 5HT₂c Receptor Activation of α₉

TABLE I

Functional reconstitution of in situ 5HT₂c receptor coupling to α₉; effect of urea extraction of membranes

Membranes were prepared from recombinant baculovirus-infected Sf9 cells (see “Experimental Procedures”). 10 µg of nonextracted membranes (60 pmol [³H]-mesulergine sites/mg) or 3.3 µg of urea-extracted membranes (180 pmol/mg) were used, respectively. In a 50-µl reaction volume, this normalized the 5HT₂c receptor concentration at 12 nM. Membranes were reconstituted with or without G-protein subunit(s) and 5HT₂c receptor ligand as indicated. Incubation time was 1 h. Numbers represent cpm bound [³⁵S]GTPγS (see “Experimental Procedures”). Values are the average of duplicate determinations, and the values in parentheses are half the difference between the duplicates.

| Subunit added | Nonextracted membranes | Urea-extracted membranes |
|---------------|-------------------------|--------------------------|
| None          | No ligand               | 1 µM 5HT                 | 1 µM mianserin           |
| α₉            | 10,759 (75)             | 10,553 (5)               | 13,102 (287)             |
| βγ            | 14,245 (260)            | 19,177 (76)              | 12,188 (123)             |
| α₉ + βγ       | 10,501 (207)            | 11,325 (348)             | 10,826 (109)             |
| M 5HT         | 18,107 (28)             | 35,043 (1401)            | 12,619 (103)             |
| M mianserin   |                         |                          |                         |
| βγ            |                          |                          |                         |
| M mianserin   |                          |                          |                         |
| M ketanserin  |                          |                          |                         |
| M mesulergine |                          |                          |                         |

Nonextracted membranes were removed from the same preparation prior to urea extraction. These membranes were washed once in solution A without urea (see “Experimental Procedures”).

The concentration of α₉ was 60 nM based on completion of α₉ GDP/GTP exchange at 1 h when reconstituted with βγ and 5HT (see Fig. 4).

The concentration of βγ is 100 nM based on amido black staining. βγ was purified from bovine rod outer segment discs (see “Experimental Procedures”).

Concentration. The concentration of 5HT that gave half-maximal activation of the 5HT₂c receptor was approximately 100 nM at both 4 and 12 nM concentrations of the 5HT₂c receptor. Also seen in this experiment, as in Table I, is a significant 5HT₂c membrane-catalyzed exchange in the absence of 5HT. This activity also increases proportionally with the membrane concentration.

The significant “basal” activity of the recombinant 5HT₂c receptor prompted us to examine antagonist pharmacology with our reconstituted system. The effects of three antagonist ligands (mianserin, ketanserin, and mesulergine) upon 5HT₂c receptor sites with the indicated concentrations of 5HT. Assays were performed as described under “Experimental Procedures.” At the highest receptor and 5HT concentrations less than 35% of the α₉ was consumed in the reaction, thus initial rates of reaction were approximated throughout.

Inhibition by antagonist ligands of 5HT-activated and basally active 5HT₂c receptor-catalyzed GDP/GTP exchange by α₉. A, the antagonism of 5HT activation of the receptor is observed for mianserin (●), ketanserin (○), and mesulergine (▲). Sf9 membranes (30 nM of 5HT₂c receptors) were incubated with 125 nM α₉, 240 nM brain βγ, 1 µM 5HT, and the indicated concentrations of antagonist. Assays were performed as described under “Experimental Procedures,” and the incubation time was 12 min. B presents inhibition of the basal catalytic activity (no 5HT added) of the 5HT₂c receptor (20 nM) with 125 nM α₉ and 320 nM brain βγ and the indicated concentrations of ketanserin (○), mianserin (●), or mesulergine (▲) and GTPγS exchange assays conducted as described under “Experimental Procedures.” For this experiment the incubation time was 90 min.
The progress analyses in the experiment of Fig. 4, we utilized 30 nM 5HT$_{2c}$ receptor with 125 nM mianserin, 250 nM brain 5HT, 100 nM mianserin, 100 µM ketanserin, or 100 µM mesulergine, or in the absence of ligand (A). At the indicated times aliquots were removed for assessment of [35S]GTP-$\gamma$S binding as described under “Experimental Procedures.”

5HT$_{2c}$ receptor, showed no inhibition of 5HT$_{2c}$ basal activity when the receptor concentration was 20 nM. This strongly suggests that antagonists for the 5HT$_{2c}$ receptor have differential efficacies as inverse agonists.

To address the molecular basis for the inhibition of “basal” activity of the 5HT$_{2c}$ receptor, we performed a progress analysis of the activation of 5HT$_{2c}$ in the absence of ligand and in the presence of saturating 5HT, mianserin, mesulergine, or ketanserin. These data are presented in Fig. 4. The progress curves were well-fit as simple exponentials. These differed significantly in rate; 50% exchange of 5HT$_{2c}$ in 5 min by the 5HT$_{2c}$-activated receptor, in 40 min by the basally active receptor, and in 2 h by the mesulergine-inhibited receptor. In the presence of mianserin or ketanserin, 5HT$_{2c}$ receptors exhibited detectable but much slower catalytic activities, turning over 25% of the 5HT$_{2c}$ receptor in 2 h. These data are consistent with general models of ligand-receptor regulation of the single rate-limiting step of GDP dissociation from 5HT$_{2c}$. In the presence of saturating concentrations of each of the three antagonists, the rates are appreciably lower than for the unoccupied receptor. The rate decrements are quite distinct for each of the antagonists, suggesting varying efficacies of inverse agonism among the three.

Based upon observations made from data shown in Fig. 3B, we first hypothesized that receptors saturated with mesulergine would have the same catalytic activity as the basal receptor, i.e. that mesulergine was a pure antagonist and thus devoid of inverse agonist properties. However, observations from the time course experiment (Fig. 4) contradicted this. It should be noted that in order to enhance the catalytic rates for the progress analyses in the experiment of Fig. 4, we utilized 30 nM receptor while only 20 nM was used for the experiments of Fig. 3. This suggested that mesulergine may be a low efficacy inhibitor of 5HT$_{2c}$ basal activity (a “partial inverse agonist”), requiring higher concentrations of receptor in order to observe its inverse agonist properties.

The experiment shown in Fig. 5 was designed to test this hypothesis. We measured the catalytic activity of the receptor in the presence or absence of saturating antagonists while varying the concentration of receptor. In the presence of 50 µM mesulergine, 5HT$_{2c}$ receptor concentrations below 30 nM appeared to catalyze 5HT$_{2c}$ GDP/GTP exchange at the same rate as the basally active receptor. However at higher concentrations of receptor, an inhibition by mesulergine was observed. Also note that ketanserin displayed slightly greater inhibition of basal activity than mianserin at saturating concentrations of ligand, consistent with its being a somewhat more efficacious inverse agonist.

**DISCUSSION**

Cells responding to a diversity of environmental stimuli utilize a repertoire of cell surface receptors and intracellular signaling proteins. Presumably, a cell employs multiple representatives from the various identified families of signal transduction proteins in responding specifically to a complex variety of stimuli. Dissection of the contributions of different receptors, G-proteins, and effector proteins to the regulation of cellular responsiveness has proven to be a formidable task (12). *In vitro* reconstitution of the various interactions between components of signal transduction cascades is one experimental approach to this question; however, the co-expression of several members of each family of proteins within any cell complicates purification of any single component as it complicates conclusive assignment of function based upon experiments in whole cells. Additionally, because the receptors and G-proteins are the first components of an amplified cascade, they are expressed in low abundance by most cells. Thus, the technical limitations of purifying sufficient quantities of receptors and G-proteins are significant hurdles to overcome for *in vitro* studies of receptor-G-protein interaction. In this report we have examined a new approach to *in situ* reconstitution of G-protein-coupled receptors that should address many of the current obstacles for investigating receptor-G-protein coupling.

First, we have developed a method of reconstituting *in situ* recombinant receptors, which has advantages over previous methods. This preparation of the receptor does not involve...
been utilized to obtain functional through other G-protein sub-families. This simplifies the reconstitution and avoids issues regarding specific chemical compositions in liposomes that may be required for proper receptor function. Additionally, we feel kinetic measurements of interactions between in situ receptors and G-protein subunits more closely reflect what occurs physiologically, due to the absence of excess phospholipid and residual detergent that may alter important hydrophobic protein-protein interactions between receptors and G-proteins.

The use of recombinant receptors has obvious advantages for characterizing important regions of molecular interaction with other proteins. Others have utilized prokaryotic expression systems (37). The baculovirus expression system offers the advantages of much higher receptor expression and post-translational protein modification. Urea extraction of nonintegral membrane proteins (60–70% of total membrane protein) reduced nonspecific GTP binding by membranes 4-fold and increased receptor site density by 3-fold. This uncoupling of the receptor allows for much greater signal to noise when reconstituting with exogenous G-protein subunits. Thus, urea-extraction of membrane preparations may be useful in searching for additional protein factors responsible for modulating receptor coupling.

Using this new system for analyzing receptor-G-protein interaction, we have established that \( \alpha_q \) purified from squid photoreceptors (26, 27) is useful for reconstitution in a mammalian PLC-linked signal transduction pathway. This gene product is expressed in high abundance in squid retina; it can be readily isolated in high yields, and it has been sequenced and cloned. The functional coupling of the 5HT\(_{2c} \) receptor with squid photoreceptor \( \alpha_q \) provides reciprocal evidence that each is a component of a PLC-\( \beta \)-mediated signal transduction pathway. The 5HT\(_{2c} \) receptor specifically interacts with \( \alpha_q \); as it does not catalyze GDP-GTP exchange on bovine \( \alpha_i \) or fractions of bovine \( \alpha_i/\alpha_q \). S99 cells express an endogenous \( \alpha_q \) that is recognized by antisera to other proteins in the family and that can activate PLC-\( \beta \) by reconstitution (36). Interestingly, the rat 5HT\(_{2c} \) receptor did not appear to couple to this protein. The recent expression and purification of four members of the \( \alpha_q \) family for reconstitution of their ability to activate PLC-\( \beta \)s (15, 36) should provide sources of related but different G-proteins that, in addition to being useful for comparison, can be genetically manipulated to examine important domains of protein-protein interaction. Squid retinal \( \alpha_q \) shares 78% sequence identity with mouse \( \alpha_q \) and \( \alpha_{13} \), and 74% identity with mouse \( \alpha_{14} \). Our data with the squid retinal \( \alpha_q \) suggest that if the mammalian proteins can be expressed with appropriate post-translational modification, our in situ reconstitution procedures should allow for the determination of differences among the \( \alpha_q \) family members. This method also should be applicable to other calcium mobilizing receptors\(^3\) as well as those coupling through other G-protein sub-families.

The recombinant baculovirus expression strategy has also been utilized to obtain functional \( \beta \gamma \) dimers (22, 38). Our studies thus far suggest that the 5HT\(_{2c}-\alpha_q \) paradigm may be useful for examination of the contribution of recombinant \( \beta \gamma \) dimers to receptor-\( \alpha \) subunit interaction. There is an increasing appreciation of the diverse roles that \( \beta \gamma \) dimers play as determinants of selectivity in cell signaling (39). We observed that a mixture of \( \beta \gamma \)s purified from bovine cortex greatly facilitated the rate of 5HT\(_{2c} \) receptor-catalyzed GTP binding by \( \alpha_q \). Interestingly, this preparation of \( \beta \gamma \) had an apparently low affinity for the 5HT\(_{2c} \) receptor, as we were unable to achieve saturation with the preparations of \( \beta \gamma \) used in these studies. This may indicate the absence of an appropriate high affinity \( \beta \gamma \) dimer in the retinal or brain preparations. Alternatively, 5HT\(_{2c} \) may have low intrinsic affinity for \( \beta \gamma \). Further studies employing recombinant \( \beta \gamma \) dimers and \( \alpha_q \) gene products may yield answers to such questions.

The ability to manipulate independently all components of the reconstitution has allowed characterization of the molecular interactions between the 5HT\(_{2c} \) receptor and squid photoreceptor \( \alpha_q \) and shown modulation of the catalytic state of the receptor by 5HT and three different antagonists. The constitutive catalytic activity of the 5HT\(_{2c} \) receptor made possible the study of inverse agonist properties of 5HT\(_{2c} \) receptor antagonists. Inhibition of basal activity of receptors is increasingly appreciated (40). It has been observed for the \( \delta \)-opioid (41), the muscarinic acetylcholine (42), the \( \beta \)-adrenergic (43), and the 5HT\(_{2c} \) (35) receptors.

Prior determination of the inverse agonist properties of 5HT\(_{2c} \) antagonists utilized studies of PI hydrolysis following stable transfection of the 5HT\(_{2c} \) DNA into the NIH 3T3 fibroblast cell line (35). Our studies expand upon these findings by examining the first biochemical step in the pathway. Even in the absence of the cascade of amplification provided in the measurement of inositol phosphates in intact cells, we find significant basal activity of the receptor and inverse agonist properties of 5HT\(_{2c} \) receptor antagonists.

Furthermore, as opposed to the intact cell approaches, our in vitro reconstitution allowed rigorous exclusion of 5HT contamination as the basis for the constitutive activity of the 5HT\(_{2c} \) receptor. Our S99 cells were cultured serum-free, and the membrane fractions we have employed were extensively washed, including with chaotrophic concentrations of urea that extracted two-thirds of the total protein in the fraction. It seems unlikely that the rapidly dissociating ligand 5HT would persist bound to the receptor after such treatment. Moreover, mesulergine did not inhibit the basal activity at low receptor concentrations, whereas mianserin and ketanserin did. If the basal activity were due to a dissociable agonist ligand, all antagonists should appear as inhibitors. The possibility that mesulergine is a weak partial agonist, exactly compensating for the displacement of contaminating 5HT, would not explain the appearance of inverse agonist activity of mesulergine at higher receptor concentrations. Indeed, the alteration of apparent intrinsic activities of partial agonists or partial inverse agonists by variation in receptor abundance is a strong prediction of currently held receptor theory (44–46). Nearly 40 years ago, it was proposed that ligand affinity and efficacy were independent parameters of receptor activation (47). For agonists with high efficacy, maximal responses were observed when the fractional occupancy of receptors was less than one. This phenomenon of "spare receptors" accounts for what we have observed with 5HT\(_{2c} \) receptor inverse agonists. It is the inverse of what one would see with a high efficacy agonist; rather than observing a maximal response until one goes below a threshold of receptor number, we see no inhibition of basal receptor activity until we supersede a receptor concentration threshold. One may imagine that the readout of this threshold (i.e. GDP-GTP exchange on \( \alpha_q \)) may vary with combinations and concentrations of \( \alpha_q \).
and βγ. Our ability systematically to vary each of the molecular components in vitro should allow for further detailed molecular analyses of receptor function at the level of G-protein activation and thus for more direct tests of existing receptor theories.

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