Cell-Specific Physical and Functional Coupling of Human 5-HT_{1A} Receptors to Inhibitory G Protein α-Subunits and Lack of Coupling to $G_{s\alpha}$†

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ABSTRACT: We have studied the physical and functional linkages of heterologously expressed human 5-HT_{1A} receptors to G protein α-subunits in HeLa and CHO-K1 cells. HeLa cells expressed immunoreactivity to G_i proteins with an apparent rank order of $G_{i\alpha3}$ (≈1 pmol/mg of protein) ≫ $G_{i\alpha1}$ (≈0.1 pmol/mg) ≫ $G_{i\alpha2}$ (<0.02 pmol/mg), whereas CHO-K1 cells expressed immunoreactivity to $G_{i\alpha2}$ (≈5 pmol/mg) ≫ $G_{i\alpha3}$ (≈0.7 pmol/mg), but not to $G_{i\alpha1}$. Both cell lines expressed large and small forms of $G_{s\alpha}$, but neither expressed detectable $G_{o\alpha}$. Agonist-promotable physical coupling of the 5-HT_{1A} receptor to G proteins was examined with high-affinity agonist binding and with co-immunoprecipitation using rabbit anti-receptor IgG fractions. Agonist treatment induced coupling of the 5-HT_{1A} receptors to G proteins with an apparent rank order of $G_{i\alpha3} > G_{i\alpha1}$, $G_{i\alpha2}$ in HeLa cells and $G_{i\alpha3} > G_{i\alpha2}$ in CHO-K1 cells. Agonist-promotable functional coupling of the 5-HT_{1A} receptors to inhibition of adenylylcyclase was measured in membranes derived from HeLa and CHO-K1 cells expressing ~2.5–3 pmol of receptors/mg of protein by preincubation with antisera raised against the carboxyl termini of the G_i protein α-subunits. A noteworthy difference between the two cell types was that antisera against the predominant G protein ($G_{i\alpha2}$) were substantially more efficacious than $G_{i\alpha3}$ antisera at blocking functional coupling to adenylylcyclase inhibition in CHO-K1 cells, whereas in HeLa cells, antisera against nonpredominant G proteins ($G_{i\alpha1}/G_{i\alpha2}$) were equally as effective as those against the predominant G protein ($G_{i\alpha3}$). No physical or functional coupling of the 5-HT_{1A} receptor to $G_{s\alpha}$ isoforms was detected in either cell line. These findings suggest that the 5-HT_{1A} receptor can physically couple to multiple distinct G_i proteins in mammalian cell membranes and that functional coupling to adenylylcyclase inhibition may be mediated by $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$. One factor influencing the relative importance of those G proteins for 5-HT_{1A} receptor-inhibited adenylylcyclase activity appears to be their relative levels of expression. Therefore, these results suggest that the coupling of the 5-HT_{1A} receptor to adenylylcyclase relies in part on factors other than its rank order of affinity for G_i protein α-subunits. It is likely that receptor/G protein molar ratios are important in determining the specificity of G protein coupling of the 5-HT_{1A} receptor.

The 5-HT_{1A}[1] receptor is an intrinsic membrane protein involved in various signal transduction cascades. However, the nature of those signaling linkages has been controversial. In hippocampus, for example, the 5-HT_{1A} receptor has been proposed to couple to the stimulation of adenylylcyclase through G_s (Shenker et al., 1985; Markstein et al., 1986), the inhibition of adenylylcyclase through pertussis toxin-sensitive G proteins (DeVivo & Maayani, 1986; Weiss et al., 1986; Bockaert et al., 1987), the inhibition of carbachol-induced stimulation of phosphoinositide turnover (Claustre et al., 1988), and the modulation of K^+ conductance and membrane hyperpolarization (Andrade et al., 1986; Colino & Haliwell, 1987; Andrade & Nicoll, 1987; Zgombick et al., 1989). Molecular cloning of the rat (Albert et al., 1990) and human (Kobilka et al., 1987; Fargin et al., 1988) 5-HT_{1A} receptors have allowed more detailed studies of signal transduction pathways linked to that receptor. In mammalian cell expression systems, the human or rat receptors inhibit adenylylcyclase (Fargin et al., 1988, 1989, 1991; Albert et al., 1990; Raymond, 1991; Liu & Albert, 1991; Raymond et al., 1992), activate or inhibit phospholipase C activity (Fargin et al., 1989, 1991; Liu & Albert, 1991; Raymond et al., 1992), increase intracellular Ca^{2+} levels (Middleton et al., 1990; Liu & Albert, 1991; Boddeke et al., 1992), inhibit BAY K8644-induced calcium influx into cells (Liu & Albert, 1991), activate protein kinase C (Raymond et al., 1989b, 1991), increase ATP-, thrombin-, and A23187-induced arachidonic acid release (Raymond et al., 1992), inhibit high conductance anion channels (Mangel et al., 1993), and activate potassium channels (Karschin et al., 1991). The 5-HT_{1A} receptor

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[1] Abbreviations: 5-HT, serotonin (5-hydroxytryptamine); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfate; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)-1,2,3,4-tetrahydronaphthalene; cAMP, adenosine 3',5'-cyclic monophosphate; GTP, guanosine triphosphate; ATP, adenosine triphosphate; PAGE, polyacrylamide gel electrophoresis; CHO-K1, chinese hamster ovary fibroblast cell; IPTG; isopropyl β-D-thiogalactopyranoside; G protein, guanine nucleotide binding regulatory protein; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; FMLP, formylmethionylleucylphenylalanine; PVDF, polyvinylidene difluoride.

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Table I: Characteristics of Antisera Used for This Study[a]

name	sequence	region	residues	specificity	source
AS/7	KENLKDCGLF	carboxy terminus	341–350	$G_{i\alpha1}$, $G_{i\alpha2}$	NEN
982	KENLKDCGLF	carboxy terminus	341–350	$G_{i\alpha1}$, $G_{i\alpha2}$	T. W. Gettys
978	LDRIAQPNYI	internal	159–168	$G_{i\alpha1}$	T. W. Gettys
J-883	CAAEEQGMLPEDLSG	internal	112–126	$G_{i\alpha2}$	S. Mumby
EC/2	KNNLKECGLY	carboxy terminus	345–354	$G_{i\alpha3}$, G_o	NEN
976	KNNLKECGLY	carboxy terminus	345–354	$G_{i\alpha3}$, G_o	T. W. Gettys
977	KNNLKECGLY	carboxy terminus	345–354	$G_{i\alpha3}$	T. W. Gettys
GO/1	ANNLRGCGLY	carboxy terminus	345–354	G_o, $G_{i\alpha3}$	NEN
U-46	NLKEDGISAAKDVK	internal	22–35	G_o	S. Mumby
RM/1	RMHLRQYELL	carboxy terminus	385–394	$G_{s\alpha}$	NEN
951	RMHLRQYELL	carboxy terminus	385–394	$G_{s\alpha}$	T. W. Gettys
985	KTREGNVRVSREL	internal	127–139	G_β	T. W. Gettys

[a] The sequences represent single amino acid codes, starting with the amino-terminal end. Residues are numbered from the initiator methionine at the amino terminus of the G protein subunit. NEN is Du Pont New England Nuclear Corporation.

produces those responses through pertussis toxin-sensitive G proteins, but the effector systems being modulated by the receptor vary from cell to cell.

The specific identity of the G protein pool(s) linking the 5-HT$_{1A}$ receptor to these signaling pathways is as yet unclear. Fargin et al. (1991) showed that $G_{i\alpha3}$ is important in both the inhibition of adenylylcyclase and the stimulation of phosphoinositide hydrolysis in HeLa cells. Strosberg's group (Bertin et al., 1992) showed that the high-affinity binding site of the human 5-HT$_{1A}$ receptors expressed in bacteria could be increased by reconstitution with a rank order of $G_{i\alpha3} > G_{i\alpha2} > G_{i\alpha1}$, but not significantly with $G_{o\alpha}$ or $G_{s\alpha}$. However, a detailed comparison of the physical and functional linkages between the 5-HT$_{1A}$ receptor and inhibitory and stimulatory G proteins in different mammalian cells has yet to be reported. The current studies examine such linkages in CHO-K1 and HeLa cells.

MATERIALS AND METHODS

Materials. [α-^{32}P]ATP (30 Ci/mmol) was from Du Pont New England Nuclear, and [^{3}H]-8-OH-DPAT (130 Ci/mmol) was from Research Products International. [^{35}S]-GTPγS was obtained from Amersham. Cell culture supplies were from Gibco. All other reagents were of the highest quality available from Sigma or Calbiochem. Antisera raised against G protein peptide sequences were obtained from the sources listed in Table I.

Cell Culture and Expression of the Human 5-HT$_{1A}$ Receptor in CHO-K1 and HeLa Cells. The receptor was stably expressed in CHO-K1 (Raymond, 1991) and HeLa (Fargin et al., 1989) cells as previously described. CHO-K1 cells were grown in Ham's F12 medium and HeLa cells in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and G-418 (400 μg/mL) at 37 °C in 95% air/5% CO_2. Levels of receptor expression in the clones used in these studies were CHO-K1–5-HT$_{1A}$/WT-26 $\approx$ 3 pmol/mg of protein and (HeLa) -HB-24 $\approx$ 2.5 pmol/mg of protein.

Protein Determinations. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Purification of Plasma Membranes. CHO-K1 or HeLa cells were washed and then homogenized in a glass Dounce homogenizer in 10 mM TES buffer (pH 7.5) containing 250 mM sucrose. A crude microsomal fraction was obtained by centrifugation at 48000g for 20 min at 4 °C. The supernatant was removed, the crude pellet was suspended in the same buffer, and purified plasma membranes were obtained by an aqueous two-phase partition as described by Morre and Morre (1989). In brief, 500 μg of crude microsomal membranes was mixed with a two-phase system (final volume $\approx$ 1.6 ml) containing 6.6% (w/w) dextran T-500, 6.6% (w/w) polyethylene glycol, 12 μM potassium phosphate (pH 7.4), and 250 mM sucrose and then centrifuged at 4 °C for 20 min at 2500g. The upper phase, containing almost exclusively plasma membranes, was removed, combined with 5 vol of ice-cold 10 mM TES (pH 7.5) containing 250 mM sucrose, and centrifuged at 48000g for 30 min. The supernatant was aspirated, and the pellet was resuspended in buffer containing 25 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, 40 μM leupeptin, and 1 μg/mL soybean trypsin inhibitor and stored at –80 °C.

Generation of Peptide Antibodies. Antiserum JWR21 was raised against a synthetic peptide derived from the predicted amino acid sequence of the putative third intracellular loop of the human 5-HT$_{1A}$ receptor (GASPAPQPKKSVNGESG-SRNWRLGVE, residues 242–267) and purified on a peptide column as previously described (Fargin et al., 1988; Raymond et al., 1989a, 1993; Raymond, 1991). Antisera were raised against the G protein carboxyl-terminal decapeptides of $G_{i\alpha3}$ and $G_{s\alpha}$ (Simonds et al., 1989; Lynch et al., 1989) and against an internal sequence (residues 159–168) of $G_{i\alpha1}$ (Goldsmith et al., 1988). Antisera that recognize $G_{i\alpha1}$ and $G_{i\alpha2}$ were raised against the carboxyl-terminal decapeptide that is shared by both of these α-subunits (Simonds et al., 1989; Lynch et al., 1989). Antisera for the β-subunits (35 and 36 kDa) were raised against a peptide sequence (residues 127–139) deduced from the human β_1 and β_2 cDNA (Fong et al., 1986; Gao et al., 1987) that had been conjugated to an octavalent polylysine base as previously described (Posnett et al., 1988). The other peptides were conjugated to KLH via a cysteine placed on the amino-terminal end of each peptide, and rabbits were immunized with each conjugate according to the method of Green et al. (1982). The antisera were characterized with respect to titer, specificity, and cross-reactivity using lysates from bacteria transformed with the genes for each of the G protein subunits (Linder & Gilman, 1991; Mumby et al., 1988; Mumby & Gilman, 1991). The antisera were used for Western blots at final dilutions of 1:16000 ($G_{s\alpha}$, 951), 1:2000 ($G_{i\alpha1}$, 978), 1:4000 ($G_{i\alpha1/2}$, 982), 1:8000 ($G_{i\alpha3}$, 977), and 1:8000 (β-common, 985) (Gettys et al., 1991). Additional antisera specific for $G_{i\alpha2}$ (J-883 against amino acids 112–126) and $G_{o\alpha}$ (U-46 against amino acids 22–35) (Mumby et al., 1988; Mumby & Gilman, 1991) were obtained from Dr. Suzie Mumby (University Texas, Southwestern Medical Center, Dallas, TX) and Du Pont New England Nuclear (see Table I).

Characterization of G Proteins in HeLa and CHO-K1 Cells. Purified plasma membranes were solubilized on ice for 1 h in 20 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, and 0.9% sodium cholate (pH 8.0). The suspension was then centrifuged at 13000*g* for 3 min and protein was assayed in the supernatant. Soluble proteins were then resolved by SDS–PAGE (12.5% acrylamide, 0.051% diallyltartardiamide) and electrophoretically transferred to Immobilon-P PVDF membranes (Millipore Corp). In some experiments, the gels were loaded with 200 μg of solubilized protein in a single lane that spanned ≈90% of the width of the gel (≈7 cm). Following the procedure of Mumby et al. (Mumby et al., 1988; Mumby & Gilman, 1991) with minor modifications (Gettys et al., 1991), PVDF membranes were blocked for 1 h at room temperature by gentle shaking with 5% dried skim milk in buffer (pH 8.0) containing 50 mM Tris, 2 mM $CaCl_2$, 80 mM NaCl, 0.02% sodium azide, and 0.2% Nonidet P-40. The membrane was then washed twice in the same low-detergent blotto and transferred into a Milliblot MP (Millipore Corp.) multiple manifold device that divided the blot into 24 separate lanes. The primary antisera were diluted in low-detergent blotto and added in replicates of four lanes for each type-specific antibody. The blot was incubated for 1 h at room temperature with gentle rocking. After three washes in low-detergent blotto, ^{125}I-labeled goat anti-rabbit IgG (1×10^6 cpm/mL) was incubated with the blot for 1 h at room temperature. The membranes were subsequently washed three times with low-detergent blotto and twice in Tris-buffered saline without skim milk, blotted dry, and exposed to Kodak XAR film with intensifying screens.

Quantitative Immunoblotting of G Proteins. Small-scale cultures of *Escherichia coli* expressing the cDNA of $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$ were grown overnight at 37 °C in Luria broth containing ampicillin (50 μg/mL). The overnight cultures were used to inoculate fresh cultures and placed at 37 °C until their optical density reached ≈0.5. The cultures were then induced for 3 h with IPTG (5 mM). Bacteria were collected from each culture and lysed, and the cell debris was removed by centrifugation. The soluble fractions were subjected to an initial DEAE–Sephacel batch purification step as described by Linder and Gilman (1991). The respective G protein α-subunits were eluted from the DEAE–Sephacel with a buffer (pH 8.0) containing 50 mM Tris, 1 mM EDTA, 1 mM DTT, 100 μM PMSF, and 300 mM NaCl. The eluted fractions were concentrated with 10 000 MW cutoff low protein binding filter units (Millipore) and stored at −80 °C. Nontransformed cultures of *E. coli* were taken through the same process described above. Saturable [^{35}S]GTPγS binding was determined by filtration through nitrocellulose filters in quintuplicate in three separate experiments. The protocol was performed exactly as described (Northup et al., 1982; Bokoch et al., 1984; Sternweis & Robishaw, 1984) using 2 μM GTPγS and 2 h incubations at 30 °C. Endogenous binding activity was subtracted from each determination after correcting for protein content. It was important to establish that the G proteins capable of binding GTPγS represented the major portion of immunoblottable G proteins.

In order to compare the amount of G protein able to bind GTPγS vs the amount of immunoblottable G protein, we performed trypsin protection assays (Itoh & Gilman, 1991) on the various partially purified G protein fractions. In that method, G protein α-subunits that are capable of binding to GTP are protected from trypsinization, yielding only a 39-kDa fragment. G proteins unable to bind to GTP are completely digested and are not detectable by immunoblot. Each preparation was incubated with 10 μM GTPγS for 60 min at 30 °C. Then, 50-μL aliquots were incubated with with 10 μL of trypsin (80 μg/mL) for 15 min at 30 °C. Reactions were stopped by adding 10 μL of soybean trypsin inhibitor (80 μg/mL). Control tubes were treated similarly, but they contained soybean trypsin inhibitor from time 0 and were kept on ice throughout. Reactions were resolved by SDS–PAGE and subjected to immunoblot and densitometry as described above.

The respective autoradiograms for each G protein were scanned by laser densitometry and the integrated volume of the bands at 39 kDa was expressed as a percentage of the volume of the bands at 41 kDa. The results of those assays showed that 99% of $G_{i\alpha1}$, 93% of $G_{i\alpha2}$, and 85% of $G_{i\alpha3}$ were protected from trypsin by GTPγS. Estimates of the amount of each G protein α-subunit in the extracts as determined by [^{35}S]GTPγS binding, corrected by the trypsin protection assays, allowed the construction of standard curves by immunoblot. Purified plasma membranes from each cell type were loaded in SDS–PAGE gels in two concentrations along with the G protein standards as described above. Autoradiograms were scanned on a densitometer (Molecular Dynamics) at 50-μm scanning resolution. Standard curves were fit to the relationship between G protein standard and band intensity, and the amounts of G protein present in the CHO-K1 or HeLa cell extracts were determined by reverse calibration. At the concentrations of G protein standards used for each G_i α-subunit, the relationship between concentration and band intensity was adequately described by a straight line.

A final consideration was that the efficiency of electrophoretic transfer might be affected by different amounts of protein in the standards and unknown samples. Because the standards contained much less protein (≪1 μg) than the unknowns (10 and 20 μg), we compared, by immunoblot, the transfer efficiency of four different amounts of standards for $G_{i\alpha1}$ and $G_{i\alpha2}$ (repeated three times for each) in the presence or absence of 20 μg of membrane proteins derived from cells deficient in $G_{i\alpha2}$ (HeLa cells) or $G_{i\alpha1}$ (CHO cells). These assays demonstrated that the transfer efficiency of the standards was only slightly impaired by the addition of membrane proteins (range 10–25%, average 15%), which could lead to an underestimation of the amount of G proteins in the unknowns by a relative increase in the immunoreactivity of the standards. Very importantly, those differences were reflected equally at each of the four concentrations of standards and represented a parallel relationship between standard curves derived in the presence and absence of supplemental membranes. Therefore, all values for membrane G proteins were adjusted upward by 15% to account for the relative transfer efficiencies.

The overall reproducibility of the immunoquantitation procedure was tested in two ways. First, the variability within each assay was typically <10% between the values determined by two different amounts of membranes (10 vs 20 μg) run on the same blot. Second, a completely separate batch of $G_{i\alpha2}$ was subjected to GTPγS binding and quantitative immunoblots, using the previous batch of $G_{i\alpha2}$ to construct a standard curve. Duplicate aliquots of ≈220 and ≈110 fmol of GTPγS binding were loaded into separate lanes and immunoblotted. The estimates from the standard curve for these two samples were ≈250 and ≈130 fmol of G protein, respectively.

Immunoprecipitation of 5-HT$_{1A}$ Receptors. Immunoprecipitations of 5-HT_{1A} receptors were performed exactly as described previously (Raymond et al., 1989a; Raymond, 1991). For co-immunoprecipitation experiments, 1×10^8 CHO-K1

cells or 5 × 10^7 HeLa cells were solubilized with CHAPS, as previously described (Raymond et al., 1989a), in the presence or absence of 1 μM 5-HT ± 10 μM spiperone as indicated. Immunoprecipitations with protein A–Sepharose beads and wash steps were carried out in the continued presence of vehicle, 1 μM 5-HT, or 1 μM 5-HT with 10 μM spiperone until two final wash steps with ice-cold 50 mM Tris (pH 7.4). G proteins were then dissociated from the immune complexes by adding 100 μL of binding buffer containing 10 μM spiperone and 10 μM GTPγS (37 °C × 1 h). The supernatants were harvested, passed sequentially over two 100-μL protein A–Sepaharose columns to remove residual IgG, and concentrated against Amicon YM-10 membranes. These products were then subjected to Western blot as described above.

Adenylylcyclase Assays. Adenylylcyclase activity in membranes was measured by the method of Salomon et al. (1974) with modifications, as previously described (Raymond et al., 1992). Antibody blocking studies were performed as outlined below.

[^{3}H]-8-OH-DPAT Binding Assays. Ligand binding was performed as previously described (Fargin et al., 1988), with modifications. After hypotonic lysis, membrane pellets were resuspended at ≈50 μg/mL in binding buffer (50 mM Tris, pH 7.4) with 1 or 2 μL of serum or buffer, as described in the figure legends, and incubated at room temperature for 45 min. After incubation, binding buffer was added to a final volume of 1 mL containing 100 pM [^{3}H]-8-OH-DPAT in the presence or absence of 10 μM spiperone or 100 μM GTP. After incubation for 30 min at 30 °C, binding assays were terminated by sequential addition of 3 mL of ice-cold binding buffer, vacuum filtration over Whatman glass fiber filters (GF/B) presoaked with 0.3% polyethylenimine, and three washes with binding buffer. Filters were added to 10 mL of scintillation fluid, and β-radiation was counted. Under these conditions, 50–90% of the specific binding typically was displaceable by GTP and, hence, represented the high-affinity agonist binding conformation. In experiments in CHO-K1 cells (n = 3, not shown), all antisera were examined for dose dependency (1:50, 1:100, 1:200, 1:500, and 1:1000 dilutions) of displacement of high-affinity binding. For AS/7, 982, 976, and EC/2, maximal displacement was attained at dilutions between 1:200 and 1:100. There was no significant increase in displacement at 1:50 dilutions of any of the antisera. Nonimmune serum and RM/1 did not significantly lower high affinity binding at any dilution. Therefore, the studies shown in Figure 2 were performed at 1:100 dilutions for all antisera.

RESULTS

Specificity of the Antisera Used for These Studies. The carboxyl-terminal regions of the G protein α-subunits are thought to contain a major portion of the receptor contact regions (Conklin & Bourne, 1993). Accordingly, strategies were devised to block coupling to receptors by using neutralizing antibodies to that portion of each G protein (Simonds et al., 1989; Conklin & Bourne, 1993). A considerable degree of homology is present between and amongst the various α-subunits, leading to concerns about the specificity of those tools. We used neutralizing sera primarily from two sources for the current studies. The first panel of sera, purchased commercially, was described by Simonds et al. (1989) and includes AS/7, EC/2, GO/1, and RM1. Antibodies from the second panel were raised in one of our laboratories (T.W.G.) using the same peptide sequences (see Table I) but a different coupling and immunization protocol. The cross-reactivity of the first panel is well-established (Simonds et al., 1989). AS/7 interacts strongly and equally well with both $G_{i\alpha1}$ and $G_{i\alpha2}$, weakly with $G_{i\alpha3}$, and not at all with $G_{o\alpha}$. EC/2 interacts strongly with $G_{i\alpha3}$ and $G_{o\alpha}$ (more so with $G_{i\alpha3}$), but also has weak cross-reactivity with both $G_{i\alpha1}$ and $G_{i\alpha2}$. GO/1 has a similar pattern of reactivity, except that it interacts more strongly with $G_{o\alpha}$ than with $G_{i\alpha3}$. RM/1 is highly specific for $G_{s\alpha}$. Because of the potential cross-reactivity, these sera typically have been used as a panel for neutralization studies and have become one of the standard tools in these types of studies. For the current work, we hoped to increase our confidence in the results by also using a second panel of sera (951, 976, 977, and 982). We tested the specificity of those sera against the various G protein α-subunits expressed in *E. coli* by immunoblot analysis and by sequential labeling with [α-^{32}P]azidoanilido-GTP (Offermans et al., 1991) and immunoprecipitation assays. The results of these assays are shown in Table II and can be summarized as follows. When used at the specified concentrations for immunoblot assays, 951 recognized only $G_{s\alpha}$ and no species of $G_{o\alpha}$ or $G_{i\alpha}$. Sera 976 and 977 recognized only $G_{i\alpha3}$, and not $G_{i\alpha1}$, $G_{i\alpha2}$, or $G_{s\alpha}$; 976 also recognized $G_{o\alpha}$ quite well, whereas 977 did not. Serum 978 was specific for $G_{i\alpha1}$; it did not recognize $G_{o\alpha}$, $G_{i\alpha2}$, $G_{i\alpha3}$, or $G_{s\alpha}$. The immunoblot cross-reactivity of J-883 has been previously published (Luthin et al., 1993); it is highly specific for $G_{i\alpha2}$, with <3% immunoreactivity with $G_{o\alpha}$, $G_{i\alpha1}$, or $G_{i\alpha3}$.

Table II: Cross-Reactivity of Some of the Antisera Used for This Study[a]

	immunoblot					immunoprecipitation				
serum	$G_{i\alpha1}$	$G_{i\alpha2}$	$G_{i\alpha3}$	$G_{s\alpha}$	n	$G_{i\alpha1}$	$G_{i\alpha2}$	$G_{i\alpha3}$	$G_{s\alpha}$	n
951	0	0	0	+	4	0	0	0	55%	2[b]
976	0	0	+	0	4	NT	NT	>90%	NT	1
977	0	0	+	0	4	0	0	>90%	0	4
978	+	0	0	0	4	+	NT	NT	0	2[b]
982	+	+	0	0	4	NT	10%	1%	0	4
J-883	2%	100%	2%	NT	6[c]	NT	NT	NT	NT	NT

[a] Immunoblots were performed as described in Materials and Methods. Unless otherwise indicated, immunoprecipitations were performed on *E. coli*-expressed recombinant subunits covalently labeled with [α-^{32}P]azidoanilido-GTP (Offermans et al., 1991) at 1:50 or 1:100 dilutions of sera. Numbers depict the percentage of immunoreactivity in the G protein columns, and the number of repetitions is under the columns headed by n. The plus signs (+) indicate that there was appreciable immunoreactivity that was not quantitated. NT means not tested. [b] These experiments were performed with HeLa or CHO cell membranes ADP-ribosylated in the presence of cholera or pertussis toxin. [c] Data for J-83 were derived from Table 2 of Luthin et al. (1993).

The specificity of some of the antisera was also tested by immunoprecipitation assays, using 1:50 or 1:100 dilutions of antisera. Those results are summarized in Table II. Sera 951 and 977 were quite efficacious and highly specific for $G_{s\alpha}$ and $G_{i\alpha3}$, respectively. Serum 982 precipitated $G_{i\alpha2}$ with ≈10-fold greater efficacy than $G_{i\alpha3}$ (10% vs 1%).

G Protein Complements in CHO-K1 and HeLa Cells. Figure 1 (top panel) illustrates the immunoreactivity detected with the various antisera used to probe purified plasma membranes from CHO-K1 cells. The first four lanes were probed with an antiserum specific for $G_{i\alpha1}$, and no immunoreactivity was detected. The second four lanes revealed a strong signal at 40–41 kDa. Because the antiserum used to probe these lanes recognizes both $G_{i\alpha1}$ and $G_{i\alpha2}$, the signal most likely is from $G_{i\alpha2}$ only. The third set of four lanes also revealed a strong signal at 41 kDa, and the specificity of the antiserum used suggests that $G_{i\alpha3}$ is present in CHO-K1 cell membranes. The fourth and fifth sets of four lanes were probed with antisera that recognized $G_{s\alpha}$ and the β-subunit common

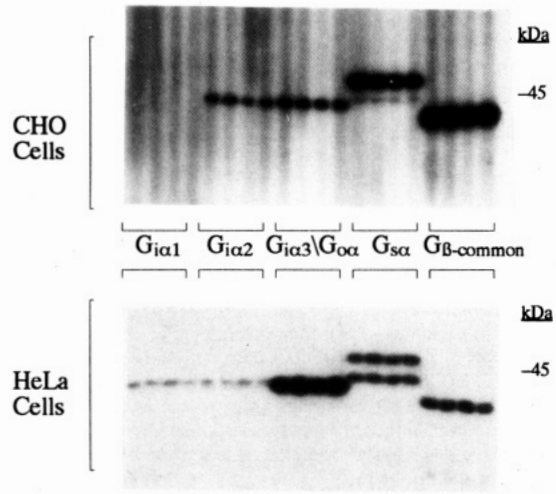

FIGURE 1: GProtein complements in purified plasma membranes from CHO-K1 and HeLa cells. Western blots were performed as described in Materials and Methods. For each gel, a 200-μg aliquot of purified plasma membranes was loaded into a single wide lane. The antisera were used for Western blots at final dilutions of 1:2000 ($G_{i\alpha1}$, 978), 1:4000 ($G_{i\alpha1/2}$, 982), 1:8000 ($G_{i\alpha3}$, 977), 1:16000 ($G_{s\alpha}$, 951), and 1:8000 (β-common, 985). The presence of very minor amounts of $G_{i\alpha2}$ (<0.015 pmol/mg of protein) in HeLa cells was confirmed with specific antiserum J-883 at a 1:500 dilution (not shown). No $G_{o\alpha}$ immunoreactivity was detected by specific antiserum U-46 (1:1000 dilution) in either cell line (not shown). The gel shown (representative of 3–10 repetitions for each serum) was subjected to autoradiography with Kodak X-AR film overnight at –80 °C.

to all G proteins, respectively. We conclude from this blot that $G_{i\alpha2}$, $G_{i\alpha3}$, $G_{s\alpha}$ (primarily the large form with lesser amounts of the small form), and the common β-subunits are expressed in CHO-K1 cells. $G_{o\alpha}$ and $G_{i\alpha1}$ were not detected; however, it is possible that they are expressed at levels below the limits of immunodetection.

The lower panel of Figure 1 illustrates the immunoreactivity detected when the various G protein antisera were used to probe plasma membranes from HeLa cells in the same format as that above. The 41-kDa band present in the first four lanes was detected with an antiserum specific for $G_{i\alpha1}$. The 40–41 kDa signal in the second four lanes suggested that $G_{i\alpha2}$ may also be present. However, because the antiserum recognizes both $G_{i\alpha1}$ and $G_{i\alpha2}$, it was possible that the antiserum was detecting only $G_{i\alpha1}$. In three separate experiments (not shown), an antiserum specific for $G_{i\alpha2}$ (J-883, 1:500 dilution) was used to probe the HeLa cell membranes and confirmed the presence of $G_{i\alpha2}$ in very minute quantities (very faint immunoreactivity was detected at about 41 kDa with prolonged exposure of the autoradiograms). The third set of four lanes also revealed a strong signal at 41 kDa, indicating the presence of $G_{i\alpha3}$ in HeLa cell membranes. The fourth and fifth sets of four lanes were probed with antisera that recognized both forms of $G_{s\alpha}$ and the β-subunit common to all G proteins. We conclude from this blot that $G_{i\alpha1}$, $G_{i\alpha3}$, $G_{s\alpha}$ (large and small forms), and common β-subunits are expressed in HeLa cells. $G_{i\alpha2}$ was present only in minute quantities. In three separate experiments, $G_{o\alpha}$ was not detected using U-46 (1:1000 dilution).

Caution is warranted when attempting to correlate apparent immunoreactivity with relative amounts of G proteins within a particular tissue. Such comparisons require that the degree of cross-reactivity, the differing titers, and the affinities of the various antisera be considered and standardized in the assay system. Therefore, to address the issue of the relative amounts of the respective $G_{i\alpha}$'s in CHO-K1 and HeLa cell membranes, an immunoblot assay using partially purified G protein

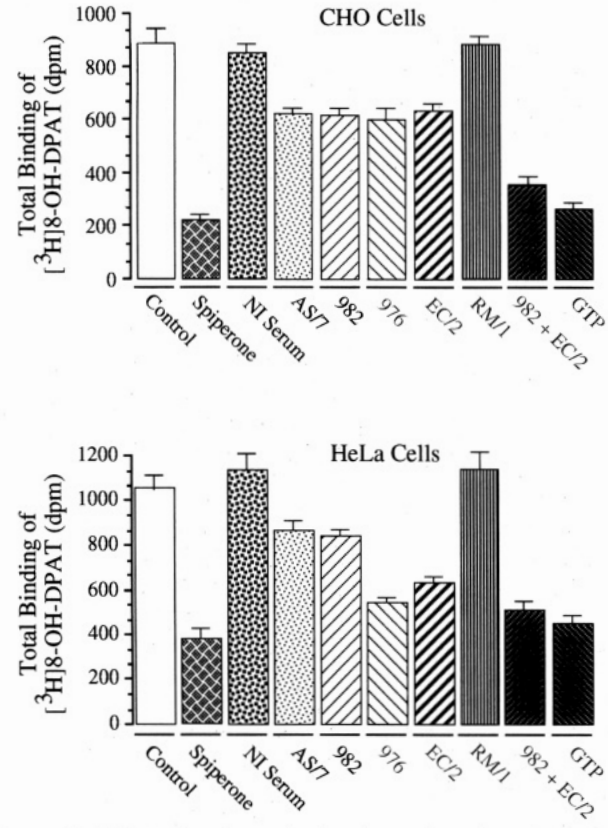

FIGURE 2: Effects of antisera raised against carboxyl terminal amino acids of various G protein α-subunits on high affinity-binding of [^{3}H]-8-OH-DPAT to the 5-HT_{1A} receptor. Experiments were performed as described in Materials and Methods at 100 pM [^{3}H]-8-OH-DPAT and 1:100 dilutions of the respective antisera. GTP and spiperone were used at 10 μM concentrations. Specificity and cross-reactivity of the sera are discussed in the text. RM/1 recognizes $G_{s\alpha}$ and, along with nonimmune serum (NI serum), serves as a control.

standards was used. In CHO-K1 cells, the following amounts of G proteins (pmol/mg of protein) were detected: $G_{i\alpha1}$ = none detected, $G_{i\alpha2}$ = 5.4 ± 0.3, and $G_{i\alpha3}$ = 0.7 ±0.01. In HeLa cells, the following amounts of G proteins (pmol/mg of protein) were detected: $G_{i\alpha1}$ = 0.1 ± 0.0, $G_{i\alpha2}$ < 0.02, and $G_{i\alpha3}$ = 1.5 ±0.1. The precision of these estimates greatly depends on the estimates of both [^{35}S]GTPγS binding, the percentage of each α-subunit protected by GTPγS from trypsin, and electrophoretic transfer efficiency. The standard errors represent only those of the final calculated values for each of the subunits and do not take into account any variability incurred at each step, which in some cases was as high as 20%. For those reasons, approximate values will be used throughout the rest of this article. Nonetheless, it is clear that these two cell lines contain different amounts and ratios of the G proteins. HeLa cells express $G_{i\alpha3} > G_{i\alpha1}$ by a factor of ≈10 with very much less $G_{i\alpha2}$, whereas CHO-K1 cells express $G_{i\alpha2} > G_{i\alpha3}$ by a factor of ≈8 with no detectable $G_{i\alpha1}$.

Effects of Antisera Raised against Carboxyl-Terminal Amino Acids of Various G Protein α-Subunits on High-Affinity Binding of [^{3}H]-8-OH-DPAT to the 5-HT_{1A} Receptor. High-affinity agonist binding is one measure of the relative amount of coupling between receptors and G proteins. As shown in Figure 2, 100 μM GTP displaced ≈85% of the specific binding (that displaced by 100 μM spiperone) of 100 pM [^{3}H]-8-OH-DPAT in both cell lines, suggesting that nearly all of the measured binding is to G protein-occupied receptors under the conditions used in this study. Antisera raised against the putative contact regions between receptor and G proteins also reduced the high affinity binding in both

cell lines. The relative contributions of $G_{i\alpha2}$ and $G_{i\alpha3}$ appeared to be approximately equal in CHO-K1 cells, as antisera to both α-subunits reduced high affinity binding by ≈35–40% each (Figure 2). The combination of antisera to $G_{i\alpha2}$ (982) and $G_{i\alpha3}$ (EC/2) resulted in an additive 75% reduction in high affinity binding in CHO-K1 cells, suggesting that both α-subunits couple to the 5-HT$_{1A}$ receptor in CHO-K1 cells and together constitute the majority of G protein α-subunits coupled to agonist-occupied 5-HT$_{1A}$ receptors in these cells. In HeLa cells, antisera to $G_{i\alpha1}/G_{i\alpha2}$ displaced only ≈20% of high-affinity binding, whereas antisera raised against $G_{i\alpha3}$ displaced 55–75% of high-affinity binding. It is noteworthy that the combination of antisera did not produce an additive inhibition of binding in HeLa cells.

The precision of those determinations relies heavily on the specificity of the antisera. It remains possible that some degree of cross-reactivity could have caused some of the displacement of high-affinity binding. Despite our efforts to quantify the degree of cross-reactivity of the various sera (Tables I and II) by immunoblot (fully denatured G proteins) and by immunoprecipitation (partially denatured G proteins), neither of those assays may adequately mimic the membrane environment (presumably minimally denatured G proteins) used for ligand binding and adenylylcyclase assays. For example, in the immunoblot assay, $G_{i\alpha1/2}$ antiserum 982 had no cross-reactivity with $G_{i\alpha3}$, whereas in the immunoprecipitation assay, it had a relatively more significant degree of cross-reactivity (≈10% of the immunoreactivity for $G_{i\alpha2}$). Moreover, 982 appeared to have only modest efficacy in the immunoprecipitation assay for $G_{i\alpha2}$. The immunoblot assay also suggested that 982 may be relatively less efficient at recognizing $G_{i\alpha2}$ than 976 is at recognizing $G_{i\alpha3}$; the CHO cell blot in Figure 1 demonstrates apparently equal immunoreactivity for $G_{i\alpha2}$ and $G_{i\alpha3}$, despite the fact that our data suggest an ≈8-fold excess of $G_{i\alpha2}$ over $G_{i\alpha3}$ in CHO cells. Therefore, it is possible that the cross-reactivity of 982 for $G_{i\alpha3}$ may be relatively important in HeLa cells, which express an excess of $G_{i\alpha3}$ over $G_{i\alpha1/2}$.

With that in mind, one possible interpretation of the results in HeLa cells is that most of the receptors couple to $G_{i\alpha3}$ and that only a small portion couples to $G_{i\alpha1/2}$. An alternative possibility is that the $G_{i\alpha1/2}$ sera might interact less specifically to neutralize a portion of the $G_{i\alpha3}$ population. Nonetheless, these findings strongly support the notion that $G_{i\alpha3}$ (when compared with $G_{i\alpha1}$ and $G_{i\alpha2}$) most likely plays a more prominent role in coupling to the 5-HT$_{1A}$ receptor in HeLa cells than in CHO-K1 cells. The relative roles for $G_{i\alpha1}$ and $G_{i\alpha2}$ are less certain in HeLa cells; however, where there appears to be much more $G_{i\alpha1}$ than $G_{i\alpha2}$. However, we still must entertain a potentially significant role for $G_{i\alpha1}$ for the inhibition of cAMP by the 5-HT$_{1A}$ receptor in HeLa cells. Nonimmune serum and an antiserum against $G_{s\alpha}$ (RM/1) had no significant effect on [^{3}H]-8-OH-DPAT binding in either cell line.

Co-immunoprecipitation of G Protein α-Subunits with an Antiserum Raised against the Human 5-HT$_{1A}$ Receptor. In order to confirm which G protein α-subunits demonstrate agonist-promotable physical association with the 5-HT$_{1A}$ receptor, liganded receptors were immunoprecipitated from CHAPS-solubilized membrane fractions derived from CHO-K1–5-HT$_{1A}$/WT-26 and -HB-24 cells. Immunoprecipitates were then probed with antisera to determine which G protein α-subunits were associated with the receptor. As expected from the data presented in Figure 2, these experiments confirmed that the 5-HT$_{1A}$ receptor is physically linked to

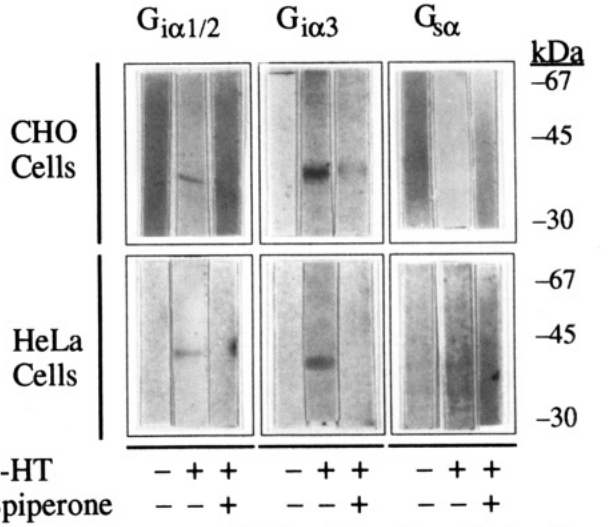

FIGURE 3: Co-immunoprecipitation of G protein α-subunits with an antiserum raised against the human 5-HT$_{1A}$ receptor. Experiments were performed as described in Materials and Methods. G protein Western blots were performed on immunoprecipitation products derived from solubilized membranes that were reacted with the 5-HT$_{1A}$ receptor antiserum JWR21, followed by precipitation with protein A–Sepharose and release of receptor-complexed G proteins by treatment of the precipitates with GTPγS and spiperone. The antisera and dilutions used were as follows: 1:4000 ($G_{i\alpha1/2}$, 982), 1:8000 ($G_{i\alpha3}$, 977), and 1:16000 ($G_{s\alpha}$, 951). The blots depicted represent three experiments for each condition. Additionally, the identity of $G_{i\alpha1}$ in immunoprecipitates from HeLa cells was confirmed once with antiserum 978 (1:2000), and the identity of $G_{i\alpha2}$ in immunoprecipitates from CHO-K1 cells was confirmed once with antiserum J-883 (1:500). Using the same sera, no $G_{i\alpha1}$ was detected in CHO-K1 cell immunoprecipitates, and no $G_{i\alpha2}$ was detected in HeLa cell immunoprecipitates. Blots were visualized by an alkaline phosphatase technique because it was much more sensitive than the ^{125}I-labeled goat anti-rabbit IgG for detecting G proteins that were co-immunoprecipitated by the 5-HT$_{1A}$ receptor antiserum JWR21.

both $G_{i\alpha2}$ and $G_{i\alpha3}$ in CHO-K1 cells in an agonist-promotable manner (Figure 3). Similar studies in HeLa cells confirmed a physical coupling to $G_{i\alpha1}$ and $G_{i\alpha3}$. There was no co-immunoprecipitation of $G_{s\alpha}$ from soluble preparations derived from either cell line under the conditions that we previously used to demonstrate a physical coupling of the α_2C10 adrenergic receptor to $G_{s\alpha}$ (Eason et al., 1992). Because these experiments were not performed with G protein standards, no estimate of the relative abundance of the immunoprecipitated G proteins should be made. However, these results clearly demonstrate that the 5-HT$_{1A}$ receptor physically couples to $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$ in an agonist-promotable manner.

Effects of Antisera Raised against Carboxyl-Terminal Amino Acids of Various G Protein α-Subunits on 5-HT$_{1A}$ Receptor-Induced Inhibition of Adenylylcyclase Activity. The previous assays reflect the state of the interaction between receptor and G protein. A functional assay of effector activity might be informative regarding the specificity of G proteins involved in distal signaling processes. As shown in Figure 4, antisera to $G_{i\alpha1}/G_{i\alpha2}$ and $G_{i\alpha3}$ reduced the ability of 5-HT to inhibit forskolin-stimulated adenylylcyclase activity in both HeLa and CHO-K1 cells. In contrast to the results obtained with agonist binding and immunoprecipitation, $G_{i\alpha1}/G_{i\alpha2}$ and $G_{i\alpha3}$ appeared to play nearly equivalent roles in mediating the inhibition of adenylylcyclase activity in HeLa cells, where the apparent immunoreactivity of $G_{i\alpha3}$ is ≈10-fold greater than $G_{i\alpha1}$ and at least 100-fold greater than $G_{i\alpha2}$. Nonspecific serum and antiserum RM/1 raised against $G_{s\alpha}$ had no significant effect. However, in CHO-K1 cells, where levels of $G_{i\alpha2}$ exceed $G_{i\alpha3}$ by ≈8-fold (and $G_{i\alpha1}$ and $G_{o\alpha}$ are not detected), $G_{i\alpha2}$

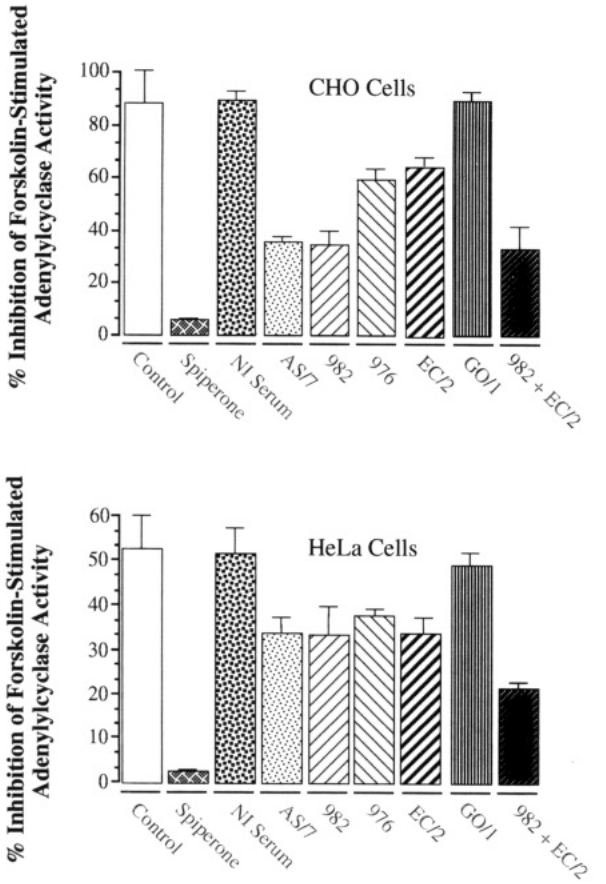

FIGURE 4: Effects of antisera raised against carboxyl terminal amino acids of various G protein α-subunits on 5-HT_{1A} receptor-induced inhibition of adenylylcyclase activity. Experiments were performed as described in Materials and Methods and the legend for Figure 2 by using preincubations with 1:100 dilutions of antisera with 5–10-μg aliquots of membrane proteins.

appeared to play a more important role in inhibiting adenylylcyclase than $G_{i\alpha 3}$. These results suggest that cell-specific factors such as the relative abundance of the G proteins may play a prominent role in receptor/G protein/effector coupling. They also suggest that the ultimate effects of a receptor on adenylylcyclase (or other effector) activity may be determined by a balance of the relative affinities of receptor for G protein and G protein for effector.

Lack of Functional Linkage of the Human 5-HT_{1A} Receptor with $G_{s\alpha}$. No definite physical or functional linkage between $G_{s\alpha}$ and the 5HT_{1A} receptor was established in CHO-K1 cells or HeLa cells. In both host cell lines, only the inhibition of basal and forskolin-stimulated adenylylcyclase activity was seen under our experimental conditions. In CHO-K1–5-HT_{1A}/WT-26 and (HeLa)-HB-24 cells, 5-HT inhibited both forskolin-stimulated adenylylcyclase activity and basal adenylylcyclase activity. No significant stimulation of adenylylcyclase activity was seen in either host cell. Treatment with pertussis toxin did not unmask a 5-HT_{1A} receptor-mediated increase in adenylylcyclase activity in either cell line. Therefore, we found no evidence of physical or functional coupling of the 5-HT_{1A} receptor to $G_{s\alpha}$ in mammalian cells in this study.

DISCUSSION

The 5-HT_{1A} receptor modulates multiple second messenger and effector systems in brain and mammalian expression systems. Most, if not all, of those linkages are mediated through pertussis toxin-sensitive G proteins. Because multiple subtypes of $G_{i\alpha}$ and $G_{o\alpha}$ are pertussis toxin sensitive, a key question about the multiple linkages is in regard to the number of G proteins that modulates the various signaling enzymes and effectors. Because the number of linkages of the 5-HT_{1A} receptor exceeds the number of known pertussis toxin-sensitive G protein α-subunits, it seemed likely that the G proteins might demonstrate some degree of promiscuity in their coupling patterns. An initial study by Fargin et al. (1991) implicated $G_{i\alpha 3}$ as the primary α-subunit linking the 5-HT_{1A} receptor to both the inhibition of adenylylcyclase and the stimulation of phosphoinositide hydrolysis in HeLa cells. Those results contrasted markedly with earlier reports that implicated $G_{i\alpha 2}$ as the primary G protein linked to the inhibition of adenylylcyclase for both α_2 adrenergic and opioid receptors (Simonds et al., 1989; McKenzie & Milligan, 1990). Subsequently, ample evidence has accumulated that α_2 adrenergic receptors can couple both physically and functionally to $G_{i\alpha 2}$ and $G_{i\alpha 3}$ in fibroblasts (McClue & Milligan, 1991; Milligan et al., 1991; MacNulty et al., 1991; McClue et al., 1992). Moreover, in CHO-K1 cells, $G_{i\alpha 2}$ and $G_{i\alpha 3}$ appear to contribute equally to the inhibition of adenylylcyclase by α_2 adrenergic receptors (Gerhardt & Neubig, 1991). Other groups have also shown that receptors which inhibit adenylylcyclase, such as somatostatin (Law et al., 1991; Murray-Whelan & Schlegel, 1992; Law et al., 1993), muscarinic (Matesic et al., 1991), adenosine (Munshi et al., 1991), and opioid (Roerig et al., 1992; Laugwitz et al., 1993) receptors, may also couple to multiple G protein α-subunits. These findings are no longer totally surprising in light of the work of Wong et al. (1992) that clearly demonstrated that $G_{i\alpha 1}$, $G_{i\alpha 2}$, and $G_{i\alpha 3}$ (as well as $G_{z\alpha}$) could all inhibit cAMP accumulation in mammalian cells. However, the issues of the relative importance of those G proteins in conferring inhibition of cAMP accumulation through specific receptors, and cellular factors that might modulate the roles of the respective G proteins, are still unresolved.

Bertin et al. (1992) recently used heterologously expressed 5-HT_{1A} receptors in *E. coli* to determine a relative rank order of affinity for this receptor to reconstituted G protein α-subunits ($G_{i\alpha 3} > G_{i\alpha 2} > G_{i\alpha 1} \gg G_{o\alpha} \gg G_{s\alpha}$). However, the physical and functional coupling parameters of the 5-HT_{1A} receptor to G proteins in mammalian cells remained to be fully elucidated. The current studies confirm that the 5-HT_{1A} receptor can also physically and functionally couple to multiple distinct G protein α-subunits in membranes derived from single cell types. They demonstrate a distinction between agonist-promotable physical coupling of the 5-HT_{1A} receptor to G proteins and its modulation of adenylylcyclase. Importantly, for the inhibition of adenylylcyclase, the relative roles of the G proteins to which the 5-HT_{1A} receptor couples appear to depend on the host cell type. Although, in our studies, the 5-HT_{1A} receptor appears to couple to G protein α-subunits with a relative rank order of $G_{i\alpha 3} > G_{i\alpha 2}$, $G_{i\alpha 1}$ and not detectably to $G_{s\alpha}$, the functional aspects of this coupling appear to be modulated by factors other than the relative coupling efficiencies of the receptor to the various G proteins. These seem to include at least the types and relative amounts of G proteins expressed in the host cells, and most likely the affinity of the activated G protein α-subunits for the effector enzyme adenylylcyclase. Other factors, including GAP-like effects that adenylylcyclase subtypes may have on specific G protein subunits, were not examined in this study.

In CHO-K1 cells, $G_{i\alpha 2}$ is expressed in $\approx$8-fold greater amounts than $G_{i\alpha 3}$ (Figure 1). These findings are consistent with previous findings described by two groups (Gerhardt &

Neubig, 1991; Dell'Acqua et al., 1993) for CHO-K1 cells, but differ somewhat for those described for CHO-DG44 cells (Law et al., 1993). The current study suggests that, in CHO-K1 cells, the 5-HT$_{1A}$ receptor physically couples to $G_{i\alpha3}$ relatively more efficiently than it does to $G_{i\alpha2}$ (Figures 2 and 3), but $G_{i\alpha2}$ appears to be more important than $G_{i\alpha3}$ in conveying 5-HT$_{1A}$ receptor-inhibited adenylylcyclase activity (Figure 4). The situation differs in HeLa cells and is confounded somewhat by the presence of $G_{i\alpha1}$ and the relatively minute amounts of $G_{i\alpha2}$. In HeLa cells, the amount of $G_{i\alpha3}$ immunoreactivity is $\approx$10-fold greater than that of $G_{i\alpha1}$ (Figure 1; Fargin et al., 1991), and the 5-HT$_{1A}$ receptor physically couples to $G_{i\alpha3} > G_{i\alpha1}/G_{i\alpha2}$ (Figure 2). However, the relative roles of $G_{i\alpha3}$ and $G_{i\alpha1}/G_{i\alpha2}$ in mediating the inhibition of adenylylcyclase activity by the 5-HT$_{1A}$ receptor seem nearly equal at 1 μM 5-HT (Figure 4). One must keep in mind that most of the current studies were performed at a single (maximal) concentration of 5-HT and single (maximal) concentrations of antisera. Although that approach has been traditionally accepted as valid, subtle differences in coupling specificity might be missed by using this approach. For example, Fargin et al. (1991) stressed the primary role of $G_{i\alpha3}$ in HeLa cells for both the inhibition of adenylylcyclase and the activation of phospholipase C by the 5-HT$_{1A}$ receptor. In contrast to the current study, theirs was performed using a submaximal concentration of 5-HT. Additionally, they did show some effects of IgG raised against $G_{i\alpha2}$ in blocking the inhibition of adenylylcyclase in HeLa cells, although their effects were less pronounced than in the current study. Another potential problem with our approach is that some degree of cross-reactivity among the antisera is possible. Nonetheless, the current data allow us to state that the 5-HT$_{1A}$ receptor can couple (at least) to $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$ in mammalian cells, depending on which α-subunit types are present in a given cell type.

We are quite certain that the 5-HT$_{1A}$ receptor can couple physically (at least) to $G_{i\alpha1}$ and $G_{i\alpha3}$ in HeLa cells and to $G_{i\alpha2}$ and $G_{i\alpha3}$ in CHO-K1 cells. Our assertions are based on the present co-immunoprecipitation studies with antireceptor sera, the present functional studies with blocking G protein antisera, and other studies using labeling of receptor-associated G proteins with the photoaffinity label [α-^{32}P]azidoanilido-GTP followed by immunoprecipitation with specific G protein antisera (T. W. Gettys, T. Fields, and J. R. Raymond, unpublished results).

Functional coupling to the various G proteins depends on other factors. These include the concentration of agonist, relative affinities for receptor/G protein interaction, affinity of effector for the various activated G protein α-subunits, the type and relative abundance of the G proteins expressed in a given cell type, and possibly influences of the effector on specific G proteins. It must be stressed that other constraints on coupling which may be operant in vivo might have been disrupted by the detergent solubilization procedure. These could include other functional constraints or physical constraints imposed by cytoskeletal components or other compartmentalization.

Therefore, functional assays may be more relevant than physical coupling experiments. Other considerations include the relative level of receptor expression as well as cell-specific expression of effector system isoenzymes (i.e., adenylylcyclases and phospholipases). This is particularly important in light of suggestions that effectors may be able to selectively activate G proteins through preferential GAP-like activity (Berstein et al., 1992; Ross & Berstein, 1993). Such considerations may account for the widely divergent patterns of coupling of an endogenous FMLP chemoattractant receptor expressed in neutrophils and the same receptor heterologously expressed in the embryonic kidney cell line 293 TSA (Uhing et al, 1992). The FMLP receptor primarily couples to phospholipases in neutrophils, but does not inhibit adenylylcyclase. However, when the receptor was expressed in 293 TSA cells, it inhibited adenylylcyclase in a pertussis toxin-sensitive manner (Uhing et al., 1992). In the latter case, the receptor numbers were approximately 10-fold higher than those in neutrophils, potentially allowing for receptor access to additional signal transduction components. It should also be noted that FMLP-induced Ca^{2+} transients lead to the activation of adenylylcyclase in neutrophils. Because FMLP does not activate adenylylcyclase in transfected 293 TSA cells, different complements of adenylylcyclase subtypes may be expressed and related to the different responses in the two cell types.

The lack of physical or functional coupling of the 5-HT$_{1A}$ receptor to $G_{s\alpha}$ is difficult to reconcile with previous reports of stimulation of adenylylcyclase by this receptor (Shenker et al., 1985; Markstein et al., 1986). In that respect, Bertin et al. (1991) have recently shown that the 5-HT$_{1A}$ receptor expressed in bacterial membranes does not increase $G_{s\alpha}$-induced GTP hydrolysis. There are several possibilities that could reconcile these divergent findings. The simplest explanation would be the existence of another 5-HT$_{1A}$-like receptor. We have recently noted the existence of such a receptor in bovine pulmonary artery smooth muscle cells (Becker et al., 1992). Another possibility may involve subtle differences in the types of $G_{s\alpha}$ expressed in each cell. There might be different physical or functional constraints (compartmentalization or cytoskeletal interactions) placed upon the receptor, $G_{s\alpha}$, or adenylylcyclase in the respective cells. A final possibility lies in the recently discovered diversity of adenylylcyclase subtypes. These subtypes demonstrate different genetic and biochemical properties and distinct anatomic patterns of expression (Krupinski et al., 1989, 1992; Tang et al., 1991; Levin et al., 1992; Katsushika et al., 1992). Of particular importance is the recent finding that both $\beta\gamma$-dimers (Federman et al., 1991) and $G_{s\alpha}$-subunits (Eason et al., 1992) are involved in α_2 adrenergic receptor-stimulated adenylylcyclase activity in CHO-K1 and other cells. Regardless, we found no evidence to support a role for the cloned human 5-HT$_{1A}$ receptor in physically and functionally coupling to $G_{s\alpha}$ in these studies.

In summary, the 5-HT$_{1A}$ human receptor expressed in mammalian cells can physically couple to multiple types of G_i α-subunits with an apparent rank order of $G_{i\alpha3} > G_{i\alpha2}$, $G_{i\alpha1}$, but not to $G_{s\alpha}$. Functional coupling to adenylylcyclase can be mediated by either $G_{i\alpha3}$ or $G_{i\alpha2}$, and possibly by $G_{i\alpha1}$. Most importantly, the relative contributions of these G proteins to 5-HT$_{1A}$ receptor-inhibited adenylylcyclase activity can vary depending on the host cell. However, it appears that $G_{i\alpha2}$ and/or $G_{i\alpha1}$ may be as important as, or relatively more important than, $G_{i\alpha3}$ in conveying 5-HT$_{1A}$ receptor-inhibited adenylylcyclase activity in some situations. These findings suggest that the determinants of specificity for the coupling of receptors to effectors through G proteins may be very complex and that there is some degreee of built-in redundancy in the coupling process between 5-HT$_{1A}$ receptors, G proteins, and effectors.

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