Selective Activation of Inhibitory G-Protein α-Subunits by Partial Agonists of the Human 5-HT_{1A} Receptor†

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ABSTRACT: Plasma membranes from Chinese hamster ovary (CHO) cells transfected with the serotonin 5-HT_{1A} receptor have been incubated with full or partial receptor agonists and the photoreactive GTP analog, 4-azidoanilido-[α-^{32}P]-GTP ([^{32}P]-AA-GTP), to characterize the resulting receptor–G-protein interactions. Subsequent solubilization and immunoprecipitation of the membranes with anti-$G_i\alpha$-2 or anti-$G_i\alpha$-3 immunoglobulins revealed that full and partial agonists produce concentration-dependent labeling of the respective G-proteins with [^{32}P]-AA-GTP. Full agonists of the 5-HT_{1A} receptor [serotonin 5-hydroxytryptamine (5-HT) and 8-hydroxy-2-(di-*n*-propylamino)tetraline (8-OH-DPAT)] produced a 7–12-fold increase in the labeling of $G_i\alpha$-2 and $G_i\alpha$-3, whereas partial agonists (rauwolscine and ipsapirone) produced a smaller incorporation (2–5-fold) of [^{32}P]-AA-GTP by the same G-proteins. The concentration of agonist producing half-maximal binding of [^{32}P]-AA-GTP by $G_i\alpha$-3 [5-HT, 48 ± 1 nM; 8-OH-DPAT, 28 ± 1 nM; ipsapirone, 22 ± 6 nM] compared to $G_i\alpha$-2 [5-HT, 124 ± 38 nM; 8-OH-DPAT, 40 ± 1 nM, ipsapirone, 82 ± 7 nM] was lower with all agonists except rauwolscine, where the EC_{50}'s were similar ($G_i\alpha$-2, 604 ± 145 nM; $G_i\alpha$-3, 708 ± 130 nM). Comparison of the rank order of agonist efficacy in producing [^{32}P]-AA-GTP binding to $G_i\alpha$-2 or $G_i\alpha$-3 with the rank order of adenylylcyclase inhibition revealed a significant correlation between the decrements in $G_i\alpha$-2 labeling and adenylylcyclase inhibition (5-HT > 8-OH-DPAT > rauwolscine > ipsapirone). In contrast, the rank order of agonist efficacy with respect to $G_i\alpha$-3 labeling (8-OH-DPAT = 5-HT > ipsapirone = rauwolscine) was weakly correlated to the efficacy of adenylylcyclase inhibition. These results are consistent with the suggestion that partial agonists of the 5-HT_{1A} receptor couple to both $G_i\alpha$-2 and $G_i\alpha$-3 with a lower efficacy than full agonists of the receptor. Perhaps more importantly, the present findings suggest that some agonists may be capable of inducing relatively selective coupling of the occupied receptor to available G-proteins.

Agonists are defined by their ability to evoke a response after binding to a receptor site, while antagonists are defined by their ability to block the effects of agonists. One of the most intriguing findings in medicinal chemistry and pharmacology is the so-called "partial agonist" ligand. These ligands typically function as agonists, often with very high potency, but act with less efficacy than classic "full agonists" with respect to effector system regulation. In the case of β-adrenergic receptors, which are thought to couple exclusively to the stimulatory G-protein family, partial agonists are unable to maximally activate the G_s-protein to the same extent as full agonists. In more complicated systems where a single receptor couples to several different G-proteins, there remains the added possibility that partial agonists induce a conformational change in the receptor that allows the recognition of only some of the G-proteins to which it is normally coupled. Over the last 2 years, it has become apparent that many receptors are capable of coupling to several distinct G-protein α-subunits within a single cell or tissue. For example, α_2-adrenergic receptors (Gerhardt & Neubig, 1991; Milligan et al., 1991; Macnulty et al., 1992), formyl peptide receptors (Gierschik et al., 1989), somatostatin (Law et al., 1991; Murray-Whelan & Schlegel, 1992), A_1 adenosine (Munshi et al., 1991), δ-opioid (Offermanns et al., 1991a; Roerig et al., 1992), and muscarinic receptors (Matesic et al., 1991) appear to couple to several different types of G_i α-subunits. In a similar fashion, full agonists can induce the human serotonin 5-HT_{1A}[1] receptor to couple to $G_i\alpha$-1, $G_i\alpha$-2, and $G_i\alpha$-3 in HeLa and CHO cells (Fargin et al., 1991; Raymond et al., 1993).

Recent work has yielded insight into the complexity of the determinants of agonism for G_i-linked receptors. It is clear that the efficacy of an agonist can be affected by the level of expression of the receptor (Peralta et al., 1988; Cotecchia et al., 1990; Fargin et al., 1989; Raymond et al., 1989; Varrault et al., 1992; Boddeke et al., 1992), the host cell type (Raymond et al., 1989, 1993; Fargin et al., 1989; Vallar et al., 1990;

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[1] Abbreviations: 5-HT, 5-hydroxytryptamine; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetraline; [^{32}P]-AA-GTP, 4-azidoanilido-[α-^{32}P]-GTP; CHO, Chinese hamster ovary; HeLa, cell line derived from cancerous cervix of Henrietta Lacks; IPTG, isopropyl β-D-thiogalactopyranoside; G-protein, guanine nucleotide binding regulatory protein, $G_i\alpha$ subunit, α-subunit of inhibitory G-protein; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; KLH, keyhole limpet hemocyanin; aa, amino acid; IgG, immunoglobulin.

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Duzic & Lanier, 1992; Uhing et al., 1992), and the type and amount of G-protein present within a cell (Duzic et al., 1992; Coupry et al., 1992; Raymond et al., 1993). The roles of cytoskeletal constraints, membrane composition or fluidity, and G-protein specificity in regulating agonist activity have yet to be explored.

In order to determine whether partial agonists selectively activate G-proteins normally coupled to full agonists, we studied the effects of two "full agonists" (5-HT and 8-OH-DPAT) and two "partial agonists" (rauwolscine and ipsapirone) (Bockaert et al., 1987; Arthur et al., 1993) with regard to their ability to induce GTP binding on $G_i\alpha$-2 and $G_i\alpha$-3 through the human 5-HT_{1A} receptor heterologously expressed in CHO cells. This was accomplished by incubation of membranes with the photoreactive GTP analog, 4-azidoanilido-[α-^{32}P]-GTP ([^{32}P]-AA-GTP), in the presence of ligand, followed by cross-linking, solubilization, and selective immunoprecipitation of $G_i\alpha$-2 and $G_i\alpha$-3. [^{32}P]-AA-GTP has been used as an affinity probe in other systems to identify specific receptor-activated G-proteins (Wange et al., 1991; Offermanns et al., 1990; Devary et al., 1987). Using this analog in the present study, it is shown that partial agonists cause the 5-HT_{1A} receptor to couple to the same G-proteins as full agonists, albeit with less efficacy. Moreover, our data suggest that some partial agonists may induce a relatively specific activation of G-protein α-subunits when compared with full agonists.

MATERIALS AND METHODS

Materials. Most materials and reagents were obtained from Sigma (St. Louis, MO). Cell culture supplies were obtained from Gibco BRL (Grand Rapids, NY). Serotonergic ligands were obtained from Research Biochemicals Inc. (Natick, MA). Azidoanilide was from Fluka Biochemicals (Ronkonkoma, NY), carbodiimide was from Bio-Rad (Melville, NY), and [α-^{32}P]-GTP was from DuPont NEN (Cambridge, MA). Affinica antibody orientation kits were from Schleicher & Schuell (Keene, NH).

Cell Lines and Receptor Expression. CHO cells were grown in monolayers at 37 °C in 95% air/5% CO_2 in Dulbecco's Modified Eagle medium supplemented with 10% fetal calf serum, penicillin (100 units/mL), and streptomycin (100 μg/mL). Stable clones were obtained by dilution cloning and selection in the presence of G-418, as previously described (Raymond, 1991; Raymond et al., 1989). A single clone that stably expresses ~0.9 pmol of 5-HT_{1A} receptor/mg of protein was chosen for studies. Cells were passaged by trypsinization and split 1:5. Confluence was usually reached in 3–5 days.

Preparation of Plasma Membranes. Cells were disrupted in a Dounce homogenizer in hypotonic buffer containing 10 mM TES (pH 7.0) and 1 mM EDTA. Unbroken cells and nuclei were removed by an initial low-speed spin at 3000*g*, and crude membranes were collected from the supernatant by a 20-min spin at 48000*g*. The pelleted membranes were resuspended in 10 mM TES (pH 7.5) containing 0.25 M sucrose, followed by the purification of plasma membranes by aqueous two-phase partition with dextran and poly(ethylene glycol), as described previously (Morre & Morre, 1989; Gettys et al., 1991). The purified plasma membranes were resuspended at 1 mg/mL in 25 mM Hepes (pH 7.4) containing 140 mM NaCl, 40 μM leupeptin, 1 μg/mL soybean trypsin inhibitor, and 1 mM EDTA and stored at −80 °C.

Antisera for Immunoprecipitation. Antisera were raised against the C-terminal decapeptide (aa 345–354) of $G_i\alpha$-3 (Goldsmith et al., 1987, 1988) and against the C-terminal decapeptide (aa 345–354) that is shared by both $G_i\alpha$-1 and $G_i\alpha$-2 (Goldsmith et al., 1987, 1988). The peptides were conjugated to keyhole limpet hemocyanin (KLH) via a cysteine placed on the N-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 cDNA for each of the G-proteins (Raymond et al., 1993; Linder & Gilman, 1991). Each antiserum was desalted using Sephadex G-25, and the G class of immunoglobulins (IgGs) from each serum was purified by HPLC using a protein A affinity column (Rainin Instrument Co, Woburn, MA). The purified IgGs were concentrated to 6 mg/mL using 10 000 MW cutoff low protein binding membranes (Millipore Corp., Bedford, MA), and 5 mg of each IgG was cross-linked to 500 μL of protein A–Sepharose beads using dimethyl suberimidate according to the manufacturer's instructions (Schleicher & Schuell, Keene, NH). The reaction was quenched, and the beads were washed and resuspended in 20 mM $KHPO_4^-$ (pH 7.0) containing 140 mM NaCl at a 1:1 ratio.

Preparation of 4-Azidoanilido-GTP. The 4-azidoanilido-[α-^{32}P]-GTP ([^{32}P]-AA-GTP) was prepared as described by Offermanns et al. (1990, 1991b), resuspended in distilled water at a concentration of 1.2 μCi/μL, and stored in the dark at −80 °C until use. The labeling of G-proteins in CHO cell membranes was essentially as described by Offermanns et al. (1991a), with slight modification. In brief, CHO cell membranes were resuspended in a buffer containing 30 mM Hepes, 100 μM EDTA, 5 mM $MgCl_2$, 1 mM benzamidine, 3 μM GDP, 100 mM NaCl, and 50 μM leupeptin (pH 7.4) at a concentration of 1.25 μg/μL. Forty microliters of membranes were placed into tubes, and 10 μL of buffer containing agonist was added to each tube. The tubes were preincubated for 10 min at 30 °C, and 10 μL of [^{32}P]-AA-GTP (0.5 μCi/tube) diluted in distilled H_2O was added to each tube followed by incubation for 10 min at 30 °C. The tubes were removed on time to an ice water bath, and the membranes were collected by centrifugation (12 000*g*). The pelleted membranes were resuspended in the same buffer described above, with the addition of 2 mM DTT, and exposed to UV light (254 nm) for 3 min at 4 °C. The membranes were collected again by centrifugation and resuspended in 125 μL of solubilization buffer containing 20 mM Tris, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, and 0.9% sodium cholate (pH 8.0). Duplicate 50-μL aliquots were removed from each tube and added to tubes containing 10 μL of protein A–Sepharose beads cross-linked to anti-$G_i\alpha$-1,2 or anti-$G_i\alpha$-3 IgG for immunoprecipitation. The tubes were incubated for 2 h at room temperature, and 200 μL of 20 mM $KHPO_4^-$ buffer containing 140 mM NaCl (PBS) was added to each tube. The Sepharose beads were collected by centrifugation and rewashed two additional times with PBS. The beads were boiled with SDS sample buffer and resolved on 12% polyacrylamide gels. The 25 μL of membranes not subjected to immunoprecipitation was also resolved on 12% gels, followed by exposure to Kodak XAR-2 film with intensifying screens. The intensity of the bands on the corresponding autoradiograms were quantitated on a Molecular Dynamics (Sunnyvale, CA) densitometer at 50-μm scanning resolution.

G-Protein Standards for Immunoprecipitation. Individual cultures of transformed *Escherichia coli* expressing the cDNA for $G_i\alpha$-2 and $G_i\alpha$-3 (Linder & Gilman, 1991) were grown overnight at 35 °C in LB containing 50 μg/mL ampicillin. 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 with 5 mM IPTG and placed in the incubator for 3 h. The cells were collected from each culture and lysed, and the cell debris was removed by centrifugation. The supernatant fractions were subjected to an initial DEAE–Sephacel 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) containing 50 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 300 mM NaCl. The eluted fractions were concentrated with 10 000 MW cutoff low protein binding filter units (Millipore Corp., Bedford, MA) and stored at –80 °C. Saturable [^{35}S]-GTPγS binding was determined in each G-protein preparation and, after subtraction of the amount of binding in a nontransformed preparation, was used as an estimate of the amount of α-subunit present in each preparation (Raymond et al., 1993).

Adenylylcyclase Assay. Adenylylcyclase activities were measured in crude membrane suspensions prepared fresh from CHO cells transfected to express the 5-HT$_{1A}$ receptor. The method was that of Salomon et al. (1974) as modified by Raymond (1991). Assay mixtures contained 20 μL of membranes and 20 μL of test mix producing final concentrations of 30 mM Tris-HCl (pH 7.2), 2 mM $MgCl_2$, 0.8 mM EDTA, 120 μM ATP with 1–2 μCi of [α-^{32}P]ATP, 50 μM GTP, 100 μM cAMP, 2.7 mM phosphoenolpyruvate, 0.2 IU pyruvate kinase, 1 IU myokinase, 0.02% ascorbate, and varying concentrations of the drugs of interest. The final assay volume was 50 μL, and the triplicates for each condition were incubated at 37 °C for 30 min. The assay was terminated by the addition of 1 mL of stop mix containing 400 μM ATP, 300 μM cAMP, and ~25 000 cpm of [^{3}H]-cAMP. cAMP was isolated from ATP by chromatography over 1 mL of Doxex and 1-mL alumina columns, and the amount of [^{32}P]-cAMP formed was determined by liquid scintillation.

Methods of Analysis. The agonist-induced labeling of G-proteins by [^{32}P]-AA-GTP was characterized using relationship functions appropriate to the sigmoidal shape of the response surface (Gettys et al., 1986, 1987, 1988). The increased labeling of G-proteins by [^{32}P]-AA-GTP in response to increasing concentrations of various 5-HT agonists was best characterized by the rising logistic ogive given as

$$y = \delta + \frac{\gamma - \delta}{1 + (x/\mu)^{\beta}} \quad (1)$$

where y is the response variable (i.e., experimental/control labeling), x is the concentration of agonist, γ is the asymptotic value of y at infinite x, δ is the asymptotic value of y at $x = 0$, μ is the concentration of agonist producing $^1/_2(\gamma - \delta) + \gamma$, and β is the standardized slope parameter where $-\beta/4\mu$ is the rate of change in the response at $x = \mu$.

Parameter estimates, along with an estimate of their standard errors, were obtained using an iterative nonlinear least-squares routine in SAS (Statistical Analysis Systems, Cary, NC). Parameter estimates obtained in the fitting process served as a basis to compare the responses to the various agonists by constructing confidence intervals around parameters of interest and conducting t-tests (Mendenhall, 1968). For example, the parameter μ represents the concentration of agonist producing a half-maximal response, while δ and γ represent the basal and maximal responses, respectively. Point estimates of maximal G-protein labeling with [^{32}P]-AA-GTP were also obtained as the mean stimulation at 10^{-5} M for each agonist and compared by a one-way analysis of variance (Cochran & Cox, 1957).

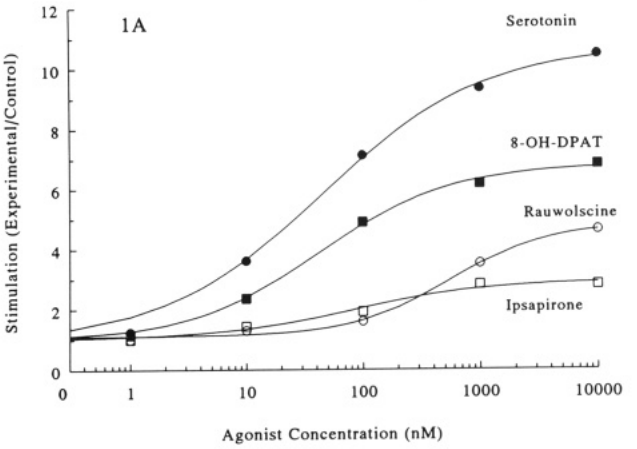

Figure 1B

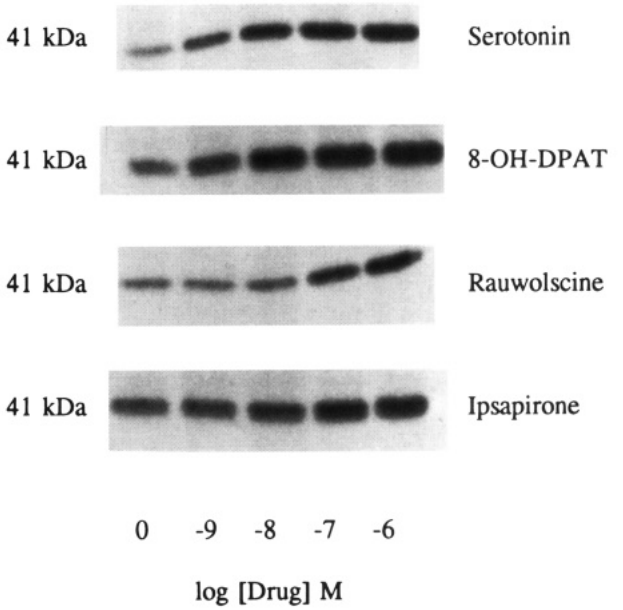

FIGURE 1: 5-HT-, 8-OH-DPAT-, rauwolscine-, and ipsapirone-stimulated binding of [^{32}P]-AA-GTP (A) to 41-kDa CHO membrane proteins. Plasma membranes (50 μg) were incubated exactly as described in "Materials and Methods" with agonist for 10 min and with [α-^{32}P]-AA-GTP for 10 min. Membranes were incubated with 0, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M each agonist, 10 μg of membranes was loaded per lane, and autoradiograms were exposed for 18 h with intensifying screens (B). The autoradiograms were scanned by laser densitometry, and the binding of [α-^{32}P]-GTP at each agonist concentration was expressed as a fraction of the control. The curves were fit by least-squares (A), and the autoradiograms (B) are representative of three experiments with each agonist.

RESULTS

Agonist-Dependent Binding of [^{32}P]-AA-GTP. Figure 1A,B illustrates that the incubation of CHO cell membranes with 5-HT, 8-OH-DPAT, rauwolscine, or ipsapirone produced a concentration-dependent increase in labeling, with [^{32}P]-AA-GTP of protein(s) migrating at 41 kDa. Expressed as a fraction of control, 5-HT produced an estimated maximal 10.6-fold increase in labeling, while a concentration of 45 ± 7 nM produced half-maximal labeling. In contrast, the partial agonist rauwolscine produced an estimated maximal increase in labeling of 4.8-fold, while the corresponding half-maximal concentration was 554 ± 151 nM (see Table 1). The maximal labeling produced by 8-OH-DPAT was lower than that produced by 5-HT ($P < 0.05$), but was greater than that produced by ipsapirone ($P < 0.05$). The full agonist, 8-OH-

Table 1: Parameter Estimates for Agonist-Dependent Labeling of G-Proteins with [^{32}P]-AA-GTP[a]

agonist	G-protein	maximal response[b]	% 5-HT response	EC_{50}[c] (nM)
5-HT	total 41 kDa	10.61 ± 0.28	100	45 ± 7
8-OH-DPAT	total 41 kDa	6.75 ± 0.22	64	42 ± 10
rauwolscine	total 41 kDa	4.77 ± 0.27	45	554 ± 151
ipsapirone	total 41 kDa	2.91 ± 0.28	27	87 ± 69
5-HT	$G_i\alpha$-2	9.33 ± 0.50	100	124 ± 38
8-OH-DPAT	$G_i\alpha$-2	7.42 ± 0.25	80	40 ± 10
rauwolscine	$G_i\alpha$-2	5.20 ± 0.28	56	604 ± 145
ipsapirone	$G_i\alpha$-2	3.01 ± 0.14	32	82 ± 7
5-HT	$G_i\alpha$-3	7.52 ± 0.22	100	48 ± 1
8-OH-DPAT	$G_i\alpha$-3	8.00 ± 0.23	106	28 ± 1
rauwolscine	$G_i\alpha$-3	4.47 ± 0.20	59	708 ± 130
ipsapirone	$G_i\alpha$-3	5.14 ± 0.17	68	22 ± 6

[a] CHO cell membranes were incubated with various concentrations of the indicated agonists in the presence of [α-^{32}P]-AA-GTP as described under Materials and Methods, and membranes were either loaded directly on SDS–PAGE gels (total 41 kDa G-proteins) or subjected to immunoprecipitation with specific antisera. The gels were dried and exposed to XAR-2 film with intensifying screens, and autoradiograms were quantitated by scanning laser densitometry. The density of each band was expressed as fold stimulation over basal labeling, and the agonist-induced labeling of the respective G-proteins was characterized by fitting the four-parameter logistic ogive given in eq 1. The parameter estimates were obtained through the fitting process for each agonist, and confidence intervals were constructed around the means and pooled standard errors of each estimate for testing hypotheses of interest. [b] The maximal response was obtained as the parameter γ in eq 1 and is an estimate of the maximal G-protein labeling with [^{32}P]-AA-GTP at infinite agonist concentration. See eq 1 in Materials and Methods. Point estimates of maximal G-protein labeling with [^{32}P]-AA-GTP were also obtained as the mean stimulation at 10^{-5} M for each agonist and were in excellent agreement with the γ parameter estimates for total 41 kDa, $G_i\alpha$-2, and $G_i\alpha$-3 (data not shown). [c] The EC_{50} is an estimate of the concentration of agonist producing half-maximal labeling of the respective G-proteins and was obtained as parameter μ in eq 1 (see Materials and Methods).

DPAT, produced a 6.8-fold increase in the labeling of the protein migrating at 41 kDa, while the partial agonist, ipsapirone, produced a 2.9-fold increase in labeling which was the lowest of the four compounds tested ($P < 0.05$). The EC_{50}'s for 8-OH-DPAT and ipsapirone were 42 ± 1 and 87 ± 69 nM, respectively (Table 1).

Immunoprecipitation of G-Protein α-Subunits. Since the type of CHO cells used (CHO-K1) has been reported to contain both $G_i\alpha$-2 and $G_i\alpha$-3 (Gerhardt & Neubig, 1991; Dell'Acqua et al., 1993; Raymond et al., 1993), it seemed likely that the 41-kDa proteins being labeled were one or both of these G-proteins. This possibility was addressed by immunoprecipitating the labeled membrane proteins with antisera specific for $G_i\alpha$-3 and $G_i\alpha$-1,2. Because little or no $G_i\alpha$-1 is expressed in CHO cells, the antiserum raised against the C-terminus of $G_i\alpha$-1 and $G_i\alpha$-2 is effectively specific for $G_i\alpha$-2 in the CHO cell (Raymond et al., 1993). Initial experiments were undertaken to establish the specificity of the immunoprecipitations. In these experiments, approximately 6–7 pmol of each recombinant G-protein α-subunit was prelabeled with [^{32}P]-AA-GTP, followed by immunoprecipitation with each IgG cross-linked to protein A–Sepharose beads. Figure 2A illustrates that anti-$G_i\alpha$-2 IgG immunoprecipitated $G_i\alpha$-2, albeit not completely, and that preadsorption of the IgG with 50 μg of the peptide, to which the antiserum was raised, effectively eliminated $G_i\alpha$-2 immunoprecipitation. Similar treatment of the labeled $G_i\alpha$-2 with anti-$G_i\alpha$-3 IgG revealed little cross-reactivity under the conditions employed (Figure 2A). Although the efficiency of immunoprecipitation was on the order of 5% in these experiments, the precision of the immunoprecipitation itself was quite good. Estimates from

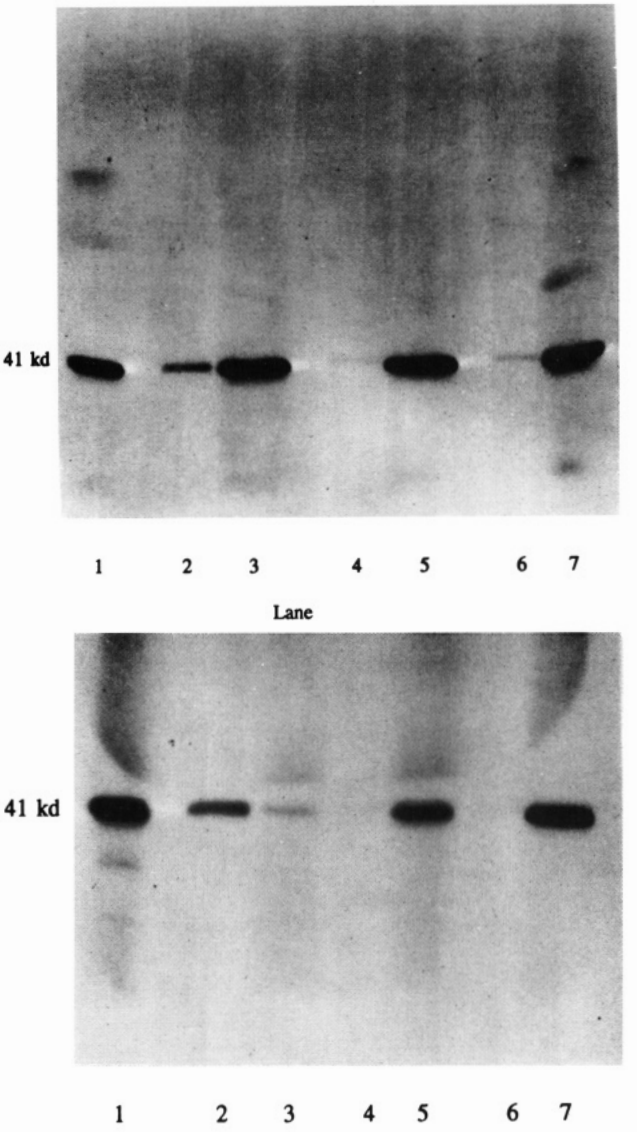

FIGURE 2: Immunoprecipitation of [^{32}P]-AA-GTP-labeled recombinant $G_i\alpha$-2 (A, top) and $G_i\alpha$-3 (B, bottom). Approximately 6 pmol of each α-subunit was incubated with [^{32}P]-AA-GTP (0.5 μCi) for 10 min in a final volume of 60 μL, as described in Materials and Methods. After cross-linking, the volume was doubled, and each α-subunit preparation was divided into four 30-μL aliquots. One aliquot from each group served as a control, while the remaining three aliquots of each group were subjected to immunoprecipitation. Panel A: lane 1, non-immunoprecipitated $G_i\alpha$-2; lane 2, pellet from IP with anti-$G_i\alpha$-2 IgG; lane 4, pellet from IP with anti-$G_i\alpha$-2 preadsorbed with 50 μg of $G_i\alpha$-2 peptide; lane 6, pellet from IP with anti-$G_i\alpha$-3 IgG; lanes 3, 5, and 7, supernatants from the pellets of lanes 2, 4, and 6. Panel B: lane 1, non-immunoprecipitated $G_i\alpha$-3; lane 2. pellet from IP with anti-$G_i\alpha$-3 IgG; lane 4, pellet from IP with anti-$G_i\alpha$-3 preadsorbed with 50 μg of $G_i\alpha$-3 peptide; lane 6, pellet from IP with anti-$G_i\alpha$-2 IgG; lanes 3, 5, and 7, supernatants from the pellets of lanes 2, 4, and 6. Dilutions of the components of each lane were made with SDS sample buffer, so that equivalent amounts of the original starting material were loaded. Autoradiograms are representative of three similar experiments.

our previous work (Raymond et al., 1993) indicate that 50 μg of CHO cell membranes would contain 20–30-fold less $G_i\alpha$-2 than used here (Figure 2A), but it should be noted that the use of excess $G_i\alpha$-2 provided a sensitive test of antiserum specificity. In subsequent experiments, it was found that the use of 10–20-fold less $G_i\alpha$-2 significantly improved the efficiency of immunoprecipitation with this antiserum (data not shown).

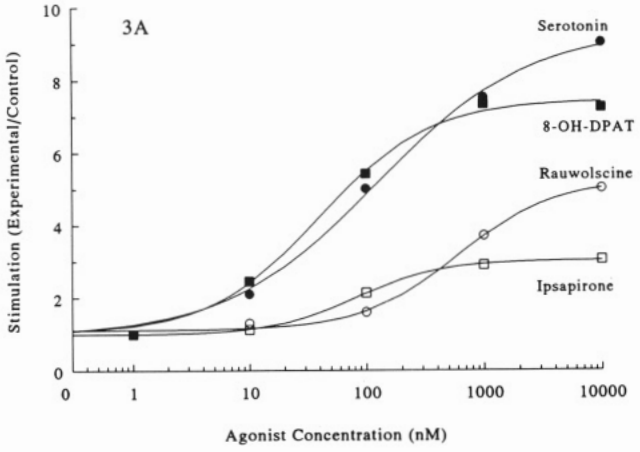

Figure 3B

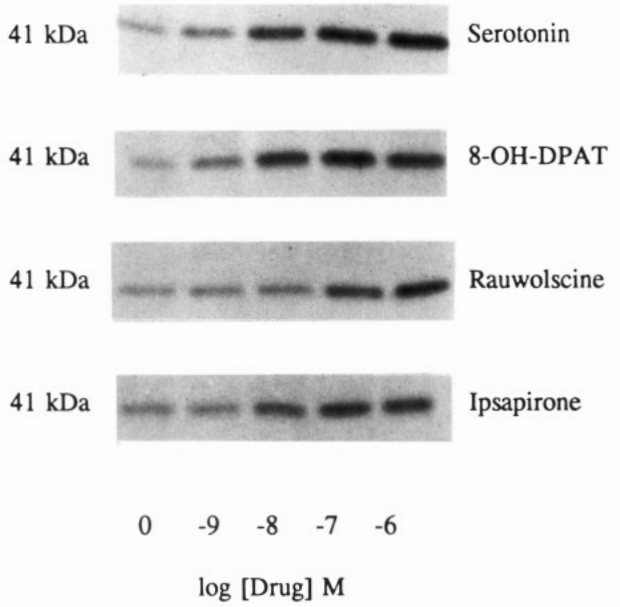

FIGURE 3: 5-HT-, 8-OH-DPAT-, rauwolscine-, and ipsapirone-stimulated labeling of $G_i\alpha$-2 (A) in CHO membranes. Plasma membranes (50 μg) were incubated exactly as described in Materials and Methods with agonist for 10 min and with [α-^{32}P]-AA-GTP for 10 min. Membranes were incubated with 0, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M each agonist, and 20-μg aliquots from each tube were incubated with anti-$G_i\alpha$-2 IgG as described in Materials and Methods. The equivalent of 15 μg of membranes was loaded per lane, and the autoradiograms were exposed for 18–36 h with intensifying screens (B). The autoradiograms were scanned by laser densitometry, and the binding of [α-^{32}P]-GTP to $G_i\alpha$-2 at each agonist concentration was expressed as a fraction of control. The curves were fit by least-squares (A), and the autoradiograms are representative of three experiments with each agonist (B).

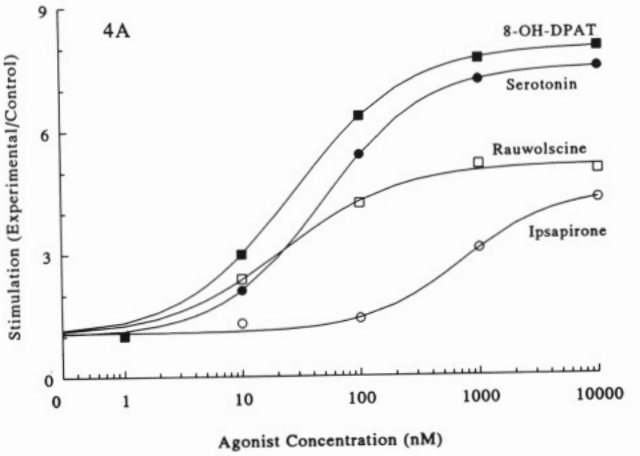

Figure 4B

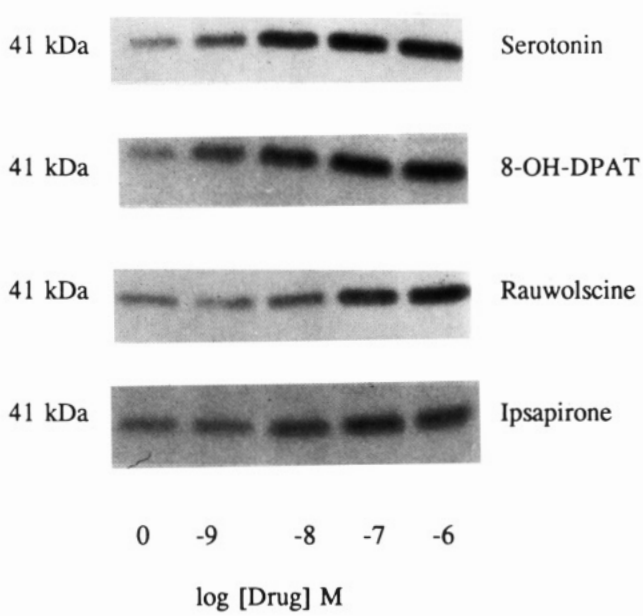

FIGURE 4: 5-HT-, 8-OH-DPAT-, rauwolscine-, and ipsapirone-stimulated labeling of $G_i\alpha$-3 (A) in CHO membranes. Plasma membranes (50 μg) were incubated exactly as described in Materials and Methods with agonist for 10 min and with [α-^{32}P]-AA-GTP for 10 min. Membranes were incubated with 0, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M each agonist, and 20-μg aliquots from each tube were incubated with anti-$G_i\alpha$-3 IgG as described in Materials and Methods. The equivalent of 15 μg of membranes was loaded per lane, and the autoradiograms were exposed for 18–36 h with intensifying screens (B). The autoradiograms were scanned by laser densitometry, and the binding of [α-^{32}P]-GTP to $G_i\alpha$-3 at each agonist concentration was expressed as a fraction of control. The curves were fit by least-squares (A), and the autoradiograms are representative of three experiments with each agonist (B).

Experiments of similar design were conducted with [^{32}P]-AA-GTP-labeled $G_i\alpha$-3, and Figure 2B reveals that anti-$G_i\alpha$-3 IgG immunoprecipitated a larger proportion of the total $G_i\alpha$-3. Subsequent experiments have suggested that the difference between the total amount of labeled $G_i\alpha$-3 (lane 1, Figure 2B) and the sum of material in lanes 2 and 3 is labeled $G_i\alpha$-3 that is loosely associated with the IP complex and is removed by subsequent washes of the pellet. Both anti-$G_i\alpha$-2 and anti-$G_i\alpha$-3 are C-terminally-directed antisera, so that the improved efficiency of immunoprecipitation by the anti-$G_i\alpha$-3 IgG may be due to its higher titer compared to the anti-$G_i\alpha$-2. Preadsorption with the $G_i\alpha$-3 peptide completely blocked immunoprecipitation, and the anti-$G_i\alpha$-2 IgG did not recognize $G_i\alpha$-3. Preliminary experiments with [^{32}P]-AA-GTP-labeled CHO cell membranes showed that anti-$G_i\alpha$-2 IgG and anti-$G_i\alpha$-3 IgG preadsorbed with their respective peptides and recombinant α-subunits were unable to immunoprecipitate their target 41-kDa proteins (data not shown). Considered together, these experiments indicate that $G_i\alpha$-2 and $G_i\alpha$-3 bind [^{32}P]-AA-GTP and that the respective antibodies against the G-proteins effectively separate these two G-proteins from one another in the immunoprecipitation protocol.

Agonist-Dependent Labeling of G-Protein α-Subunits. Figure 3 indicates that 5-HT and rauwolscine produced concentration-dependent labeling of $G_i\alpha$-2 in CHO membranes. As with total 41-kDa G-proteins (Figure 1), 5-HT was almost twice as efficacious (9.33-fold vs 5.2-fold) as rauwolscine in increasing [^{32}P]-AA-GTP incorporation ($P <$

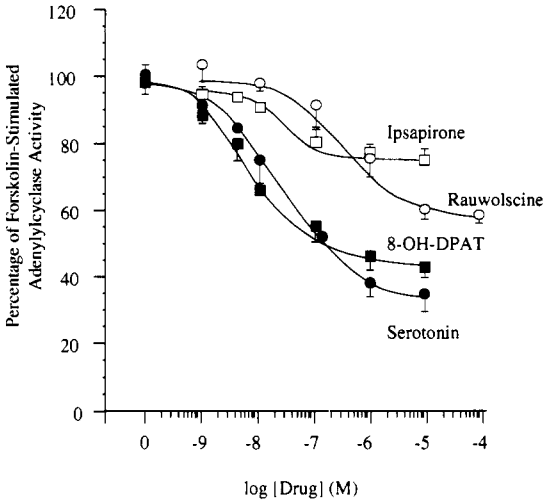

FIGURE 5: Inhibition of adenylylcyclase activity by 5-HT agonists in membranes from CHO cells transfected with the 5-HT_{1A} receptor. Adenylylcyclase activity was measured as described in Materials and Methods in the presence of vehicle (basal) or 100 μM forskolin, and in the presence of various concentrations of the agonists. Basal activity averaged ~5 nmol of cAMP/mg of protein/30 min and was stimulated 15–20-fold in the presence of forskolin. This representative experiment was replicated five times, and the curves were fit by least-squares analysis.

0.05). The EC_{50} for $G_i\alpha$-2 labeling by 5-HT was 124 ± 38 nM, while the corresponding estimate for rauwolscine was 604 ± 145 nM (Table 1). Examination of Figure 4 indicates that 5-HT and rauwolscine also produced concentration-dependent labeling of $G_i\alpha$-3 in CHO cell membranes and, as with $G_i\alpha$-2, 5-HT was nearly twice as efficacious as rauwolscine (7.5-fold vs 4.5-fold, $P < 0.05$). The concentration of 5-HT producing half-maximal labeling of $G_i\alpha$-3 was 48 ± 1 nM, while the corresponding estimate for rauwolscine was 708 ± 130 nM. It is interesting that EC_{50}'s for $G_i\alpha$-2 and $G_i\alpha$-3 labeling by rauwolscine are similar, while the EC_{50} for $G_i\alpha$-3 labeling by 5-HT was ~2-fold lower than the estimate for $G_i\alpha$-2.

Figures 3 and 4 indicate that 8-OH-DPAT and ipsapirone also produced concentration-dependent labeling of $G_i\alpha$-2 and $G_i\alpha$-3, respectively. In both cases, the full agonist, 8-OH-DPAT, produced significantly ($P < 0.05$) greater incorporation of [^{32}P]-AA-GTP into the respective G-proteins than did the partial agonist, ipsapirone ($G_i\alpha$-2, 7.4-fold vs 3.0-fold; $G_i\alpha$-3, 8.0-fold vs 5.1-fold). It is particularly interesting that, relative to 5-HT, 8-OH-DPAT acted as a full agonist with respect to $G_i\alpha$-3 labeling and as a partial agonist with respect to $G_i\alpha$-2 labeling ($P < 0.10$). This question was also addressed by comparing point estimates of $G_i\alpha$-2 labeling produced by 10 μM 5-HT and 10 μM 8-OH-DPAT and revealed that 5-HT was significantly more efficacious than 8-OH-DPAT ($P < 0.05$). Most importantly, the efficacy of ipsapirone to increase the labeling of $G_i\alpha$-3 was clearly greater than its ability to increase the labeling of $G_i\alpha$-2 ($P < 0.05$), in which case the efficacy of rauwolscine (5.20 ± 0.28) was greater than that of ipsapirone (3.01 ± 0.14, $P < 0.05$). This was particularly apparent when the data were expressed as ratios of the maximal effect of 5-HT. The EC_{50}'s for the 8-OH-DPAT and ipsapirone in the case of $G_i\alpha$-2 were 40 ± 10 and 82 ± 7 nM, while the corresponding estimates for $G_i\alpha$-3 were 28 ± 1 and 22 ± 6 nM, respectively (Table 1).

Inhibition of Adenylylcyclase. Incubation of CHO cell membranes with 100 μM forskolin in the presence of various concentrations of 5-HT, 8-OH-DPAT, rauwolscine, or ipsapirone produced concentration-dependent inhibition of adenylylcyclase activity with all agonists (Figure 5). Estimates of the EC_{50}'s and maximal responses were obtained by curve fitting (Table 2) and illustrate the expected stratification in efficacy with 5-HT ≥ 8-OH-DPAT ≥ rauwolscine > ipsapirone (Raymond et al., 1992; Arthur et al., 1993). The full agonist, 8-OH-DPAT, was the most potent among the compounds tested, while the partial agonist, rauwolscine, had the lowest potency yet was only slightly less efficacious than 8-OH-DPAT with respect to adenylylcyclase inhibition (Table 2). Ipsapirone best illustrates the lack of correspondence between potency and efficacy in the sense that its EC_{50} was similar to those of full agonists, while its efficacy was only a fraction of that seen for 5-HT (Table 2). Taken together, the inhibition curves exhibit the noted characteristics of full and partial agonists, namely, variations in efficacy that are not necessarily related to potency.

Table 2: Inhibition of Adenylylcyclase in CHO Cells by Agonists of the 5-HT_{1A} Receptor[a]

agonist	EC_{50} (nM)	% maximal inhibition	% 5-HT response
5-HT	166 ± 32	63 ± 3	100
8-OH-DPAT	78 ± 12	54 ± 2	86
rauwolscine	1500 ± 200	46 ± 3	73
ipsapirone	130 ± 14	19 ± 4	30

[a] Adenylylcyclase assays were performed in CHO cell membranes in triplicate in the presence of 100 μM forskolin as previously described (Raymond, 1991). The experiments were replicated five times for each compound, and basal activity was subtracted from each determination to calculate % inhibition. The means and standard errors for % maximal inhibition were pooled from the five experimental replicates.

Relation of Adenylylcyclase Inhibition to G-Protein Subunit Labeling. Comparison of the rank order of potency for the four compounds with respect to the activation of $G_i\alpha$-2 (8-OH-DPAT > ipsapirone ≥ 5-HT > rauwolscine) and $G_i\alpha$-3 (ipsapirone ≥ 8-OH-DPAT > 5-HT > rauwolscine) suggested that each G-protein subtype may have its own distinctive pharmacological profile with reference to the 5-HT_{1A} receptor subtype (Table 1). Notably, comparison of the efficacy of these agents to maximally activate each G-protein, made by contrasting the maximal efficacy of each compound with respect to 5-HT and expressed as an agonist activity ratio (% of 5-HT response; see Table 1), revealed distinct patterns of activation. For rauwolscine, the ratios were similar between $G_i\alpha$-2 (56%) and $G_i\alpha$-3 (59%). However, 8-OH-DPAT appeared to be a full agonist for $G_i\alpha$-3 and a partial agonist for $G_i\alpha$-2 (Table 1). Perhaps most striking was the observation that ipsapirone displayed the most disparate agonist ratios between $G_i\alpha$-2 (32%) and $G_i\alpha$-3 (68%). These results clearly demonstrate that a panel of agonists, acting through a single receptor type, can differentially activate $G_i\alpha$ subtypes. Examination of the relationship between the efficacy of activation of $G_i\alpha$ subtypes ($G_i\alpha$-2 and $G_i\alpha$-3) and the efficacy of adenylylcyclase inhibition suggested that $G_i\alpha$-2 activation was closely related to adenylylcyclase inhibition by the 5-HT_{1A} receptor. For example, there was a strong correlation between the efficacy of adenylylcyclase inhibition and $G_i\alpha$-2 labeling with [^{32}P]-AA-GTP ($P > 0.04$, $R^2 = 0.92$). In contrast, the correlation between the efficacy of adenylylcyclase inhibition and $G_i\alpha$-3 labeling with [^{32}P]-AA-GTP was not significant ($P > 0.34$, $R^2 = 0.44$; see Table 1).

DISCUSSION

The complex nature of signaling through G-proteins has been clearly demonstrated by recent molecular cloning and biochemistry studies. These studies have delineated a surprising degree of promiscuity for the coupling of single receptor

types to specific G-protein subunits and second messenger pathways (Wong et al., 1992; Law et al., 1991; Roerig et al., 1992; Murray-Whelan & Schlegel, 1992; Matesic et al., 1991; Munshi et al., 1991; Roerig et al., 1992; Laugwitz et al., 1993). The ability of a compound to activate or inhibit these second messenger pathways after binding to a receptor defines such compounds as agonists. Antagonists are defined by their ability to block the effects of agonists. A very unusual and intriguing class of compounds has been described as "partial agonist" ligands, which activate or inhibit second messenger pathways with less efficacy than classical full agonists. The molecular basis of partial agonism is poorly understood, particularly for receptors like the 5-HT$_{1A}$ receptor which couple to more than one $G_i\alpha$ subunit in mammalian cells (Fargin et al., 1991; Raymond et al., 1993; Bertin et al., 1992). One possible explanation is that partial agonists such as ipsapirone (Bockaert et al., 1987) and rauwolscine (Arthur et al., 1993) are generally less able to activate all of the types of $G_i\alpha$ subunits to which the receptor is normally coupled. However, that explanation does not account for the observation that 8-OH-DPAT is a full agonist in some systems and a partial agonist in others (Varrault & Bockaert, 1992). A second explanation is that there is a selective inability of those ligands to activate one or more of the normally targeted α-subunits. These subunits, in turn, may have specific roles in modulating effectors, and some agents may be full agonists for one effector, but only partial agonists for another.

Recent studies with recombinant 5-HT$_{1A}$ receptors expressed in various mammalian host cell lines have shed some light on this issue (Fargin et al., 1989, 1991; Raymond et al., 1989, 1993; Varrault et al., 1992; Boddeke et al., 1992; Middleton et al., 1990; Liu & Albert, 1991; Karschin et al., 1991). For example, two groups have shown that alterations in the number of 5-HT$_{1A}$ receptors affect the maximum degree to which 5-HT is capable of inhibiting adenylylcyclase, but not its potency (Varrault et al., 1992; Yocca et al., 1992). In HeLa cells, an increase in the amount of expressed receptors increases the efficacy of some partial agonists to increase intracellular calcium levels (Boddeke et al., 1992). Finally, a reduction in the availability of G-proteins by pertussis toxin treatment of CHO cells transfected with the 5-HT$_{1A}$ receptor reduced both the efficacy and the potency of 5-HT to inhibit adenylylcyclase (Raymond et al., 1992). Some, but not all aspects of those findings can be explained by the classical (Kenakin & Morgan, 1989; Keen, 1988) or operational (Keen, 1988; Black et al., 1985) models of agonism. One of the difficulties in interpreting those studies in the context of agonism models has been the inability to measure the effects of agonists on the activity of individual G-protein subtypes. Prior to the current study and one by Laugwitz et al. (1993), the effects of receptor agonists on individual G-protein activities were primarily confined to reconstituted systems.

The ability to directly measure G-protein activation by photoaffinity labeling with [^{32}P]-AA-GTP, followed by the separation of individual G-protein α-subunits, provided the tools to perform such an evaluation. The current studies demonstrate that partial agonists of the 5-HT$_{1A}$ receptor produced decreased labeling of both $G_i\alpha$-2 and $G_i\alpha$-3 when compared to full agonists such as 8-OH-DPAT and 5-HT. Thus, it appears likely that partial agonists of the 5-HT$_{1A}$ receptor may induce a structural conformation less capable of G-protein coupling and/or activation. Rauwolscine appeared to have equal impairment in its ability to activate both $G_i\alpha$-2 and $G_i\alpha$-3 when compared with 5-HT. The most intriguing finding is that ipsapirone was clearly less efficacious in activating $G_i\alpha$-2 than $G_i\alpha$-3. Differences in $G_i\alpha$-2 and $G_i\alpha$-3 labeling are not due to the variable ability of these proteins to bind [^{32}P]-AA-GTP, as both bind the analog with similar affinities at a given Mg^{2+} concentration (T. A. Fields, M. E. Linder, and P. J. Casey, unpublished observations). These results suggest that intrinsic properties of the partial agonists themselves working through receptors may function to activate differentially various G-protein α-subunits independent of membrane properties or constraints. Therefore, the basis of partial agonism for some ligands of the 5-HT$_{1A}$ or other receptors may reside in highly selective uncoupling from a single type of G-protein α-subunit. For example, these results might explain why 8-OH-DPAT is a full agonist in some systems and a partial agonist in others (Varrault & Bockaert, 1992). The present results suggest that, in systems where $G_i\alpha$-3 is the most important modulator of an effector, 8-OH-DPAT would be a full agonist, whereas in systems where $G_i\alpha$-2 is critical, the compound would be a partial agonist.

One intriguing finding is that the pharmacological profile of the agents tested was more similar for the inhibition of adenylylcyclase and activation of $G_i\alpha$-2 than for the activation of $G_i\alpha$-3 in CHO cells. This similarity was apparent for the rank order of efficacy, as well as the magnitudes of agonist ratios when compared to 5-HT. Although the observation does not prove a definitive role for $G_i\alpha$-2 in 5-HT$_{1A}$ receptor-mediated inhibition of adenylylcyclase, it is consistent with our previous work using antibody trapping of specific G_i subtypes in 5-HT$_{1A}$-transfected CHO cells (Raymond et al., 1993). That work demonstrated that trapping of $G_i\alpha$-2 or $G_i\alpha$-3 could reduce high-affinity binding to the receptor, but trapping of $G_i\alpha$-2 produced near-total blocking of 5-HT-mediated inhibition of adenylylcyclase (Raymond et al., 1993). Thus, both studies, using different experimental approaches, support the suggestion that 5-HT$_{1A}$ receptors activate both $G_i\alpha$-2 and $G_i\alpha$-3, but activated $G_i\alpha$-2 is the primary conveyor of the signal to inhibit adenylylcyclase. In contrast to the current work, Fargin et al. (1991) suggested that $G_i\alpha$-3 was the primary mediator of adenylylcyclase inhibition by the 5-HT$_{1A}$ receptor in HeLa cells. However, HeLa cell membranes contain very little $G_i\alpha$-2 relative to $G_i\alpha$-3, while in CHO cells the ratio of $G_i\alpha$-2 to $G_i\alpha$-3 is on the order of 9:1 (Raymond et al., 1993). Taken together, previous (Fargin et al., 1991) and current works support the hypothesis that the relative importance of different G-protein α-subunits in coupling the 5-HT$_{1A}$ receptor to effector systems may vary depending on the cell type (Liu & Albert, 1991).

In aggregate, our findings could have substantial physiological significance for receptors that couple to multiple G-proteins and signal transduction pathways in a single cell. Partial agonists could provide the specific means to alter the coupling of a receptor so that only a single species of its cognate G-proteins is activated. The most exciting implication of this work is that selective G-protein-activating properties of partial agonists could lead to the development of therapeutic compounds with dramatically reduced side effects.

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