Antibody Capture Assay Reveals Bell-Shaped Concentration-Response Isotherms for h5-HT$_{1A}$ Receptor-Mediated G$\alpha_{i3}$ Activation: Conformational Selection by High-Efficacy Agonists, and Relationship to Trafficking of Receptor Signaling

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Received April 4, 2002; accepted May 23, 2002 This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Although serotonin 5-HT$_{1A}$ receptors couple to several Gi/o G-protein subtypes, little is known concerning their differential activation patterns. In this study, in membranes of Chinese hamster ovary cells expressing h5-hydroxytryptamine$_{1A}$ receptors (CHO-h5-HT$_{1A}$), isotherms of 5-HT-stimulated guanosine-5'-O-(3-[35S]thio)-triphosphate ([35S]GTP$\gamma$S) binding were biphasic, suggesting coupling to multiple G-protein subtypes. The high potency component was abolished by preincubation with an antibody recognizing G$\alpha_{i3}$ subunits and was resistant to induction of $^{35}$S]GTP$\gamma$S dissociation by unlabelled GTP$\gamma$S, thus yielding a bell-shaped concentration-response isotherm. To directly investigate G$\alpha_{i3}$ activation, we adopted an antibody-capture/scintillation proximity assay. 5-HT and other high-efficacy agonists yielded bell-shaped [35S]GTP$\gamma$S binding isotherms, with peaks at nanomolar concentrations. As drug concentrations increased, G$\alpha_{i3}$ stimulation progressively returned to basal values. In contrast, the partial agonists (-)-pinadolol and 4-(benzodioxan-5-yl)-1-(indan-2-yl)piperazine (S15535) displayed sigmoidal stimulation isotherms, whereas spiperone and other inverse agonists sigmoidally inhibited [35S]GTP$\gamma$S binding. Agonist-induced stimulation and inverse agonist-induced inhibition of G$\alpha_{i3}$ activation were abolished by pretreatment of CHO-h5-HT$_{1A}$ cells with pertussis toxin. The high potency agonist (N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl)-cyclohexane-carboxamide) fumarate (WAY100,635), and absence of G$\alpha_{i3}$ coupling. Taken together, these data show that low but not high concentrations of high-efficacy 5-HT$_{1A}$ agonists direct receptor signaling to G$\alpha_{i3}$. In contrast, partial agonists favor h5-HT$_{1A}$ receptor signaling to G$\alpha_{i2}$ over a wide concentration range, whereas inverse agonists inhibit constitutive G$\alpha_{i3}$ activation.

Serotonin 5-HT$_{1A}$ receptors are therapeutic targets in the management of anxiety, depression, and schizophrenia (Barnes and Sharp, 1999; Millan, 2000). Cloning of human 5-HT$_{1A}$ receptors and their expression in recombinant cell lines has permitted the elucidation of their signal transduction pathways. They couple, thus, to multiple transduction pathways, including adenyl cyclase, phospholipase C, sodium-dependent phosphate uptake, protein kinase C, Na$^+$/K$^+$ ATPase, K$^+$ channels, and mitogen-activated protein kinase (Fargin et al., 1989; for reviews, see Gerhardt and Heerikhuizen, 1997; Raymond et al., 1999). These cellular responses are abolished by pretreatment with Bordetella pertussis toxin (PTX), which ADP-ribosylates Ga subunits of the Gi/o family, indicating the coupling of 5-HT$_{1A}$ receptors to Gi/o-proteins. Subtypes of Ga subunits engaged by 5-HT$_{1A}$ receptors have been identified by coexpression studies in a variety of bacterial, insect, and mammalian expression systems (for review, see Raymond et al., 1999). Thus, reconstitution of h5-HT$_{1A}$ receptors expressed in Escherichia coli with recombinant G$\alpha_{i2}$ subunits most markedly increased affinity for the agonist [3H](-)-8-hydroxy-dipropyl-aminotetralin ([3H]8-OH- DPAT), followed by G$\alpha_{i2}$ and G$\alpha_{i1}$ subunits (Bertin et al.,

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; PTX, Bordetella pertussis toxin; 8-OH-DPAT, (+)-8-hydroxy-dipropyl-aminotetralin; CHO, Chinese hamster ovary; CHO-h5-HT$_{1A}$, Chinese hamster ovary cells expressing human 5-HT$_{1A}$ receptors; GPCR, G-protein coupled receptor; SPA, scintillation proximity assay; [35S]GTP$\gamma$S, guanosine-5'-O-(3-[35S]thio)-triphosphate; S15535, 4-(benzodioxan-5-yl)-1-(indan-2-yl)piperazine; WAY100,635, (N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyridinyl)-cyclohexane-carboxamide) fumarate; 5-CT, 5-carboxamidotryptamine; S16924, [3H]-2-[2-(2,3-dihydro-benzo[1,4]dioxin-5-ylxylo)-ethyl]-pyrrolidin-3-yl)-1-(4-fluoro-phenyl)-ethanone] HCl; S14671, [3H]-2-[4-(fluorobenzoylamino)ethyl]-4-(7-methoxynaphthyl)piperazine] HCl; S14761, [1-(7-methoxynaphthyl)-1-yl]-4-[2-(2-thenoylamino)-ethyl]piperazine] HCl; UK14,304, 5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinolnaline; RGS, regulator of G-protein signaling; Ro318220, 3-[1-[3-(amidinothio)propyl]-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide methanesulfonate; pERK, phosphorylated extracellular signal regulated kinase.
A similar reconstitution strategy in insect Spodoptera frugiperda (S/B) cells likewise showed coupling of h5-HT1A receptors to G/o G-protein subtypes, and particularly to Gaq (Butkerait et al., 1995; Clawges et al., 1997). Functional responses of h5-HT1A receptors have also been shown to be mediated by Gaq and Gai2 subunits. Thus, microphysiometric measurement of medium acidification showed that PTX-resistant mutants of Gai2 or Gai2 "rescued" agonist-induced Na+/H+ exchange in h5-HT1A receptor-expressing Chinese hamster ovary (CHO) cells pretreated with PTX (Ganovskaya et al., 1997). Furthermore, antisera raised against Gaq and Gai2 subunits attenuated h5-HT1A receptor-mediated inhibition of adenylyl cyclase activity in CHO and HeLa cells (Raymond et al., 1993). Taken together, these studies converge toward the conclusion that 5-HT1A receptors couple preferentially to Gai2 subunits, followed by Gai2 and, less strongly, Gao and Gaq subunits (Clawges et al., 1997). On the other hand, coupling of 5-HT1A receptors to Goq and Gao is weak or absent (Raymond et al., 1993). Nevertheless, coupling to G-protein subtypes can be investigated when receptor/G-protein stoichiometry is constrained [e.g., in h5-HT1A-Gao fusion proteins (Kellett et al., 1999)], and a recent study reported stimulation of adenylyl cyclase by 5-HT1A receptors at high agonist concentrations, suggesting that Gs activation is, in fact, possible under certain conditions (Malmberg and Strange, 2000).

The issue of differential coupling to G-proteins is of particular interest in view of accumulating reports describing agonist-directed trafficking of receptor signaling (Kenakin, 1995). Thus G-protein-coupled receptors (GPCRs) may exhibit differential coupling to intracellular G-proteins or second messenger systems depending on the agonist employed. For example, agonists at h5-HT2C receptors display differential orders of efficacy for activation of phospholipase C versus phospholipase A2 (Berg et al., 1998; for review, see Clarke and Bond, 1998), whereas agonists differentially induce coupling of h5-HT2C-adrenoceptors to Gi- or Gs-mediated modulation of adenylyl cyclase activity (Brink et al., 2000). In the case of h5-HT1A receptors, Gettys et al. (1994a) undertook a limited study of the potency of different agonists in activating Gaq and Gai2 subunits, as detected by 4-azidoanilido-[α-32P]GTP labeling, membrane solubilization, and immunoprecipitation of Ga subunits. They showed that, whereas some agonists (5-HT and 8-OH-DPAT) more potently activated Gaq than Gai2, rauwolscine activated both subunits with similar potency (Gettys et al., 1994a). However, this technique is cumbersome and ill-adapted to broad investigation of numerous drugs and assay conditions. Furthermore, the molecular basis for these examples of agonist-directed trafficking are little characterized and are probably influenced by multiple factors, including receptor and G-protein expression levels, ionic conditions, etc.

In view of the above considerations, the present study employed a recently developed antibody-capture technique coupled to scintillation proximity assay (SPA) detection (DeLapp et al., 1999) to characterize Gaq subunit activation at recombinant human 5-HT1A receptors stably expressed in Chinese hamster ovary cells (CHO-h5-HT1A cells; Newman-Tancredi et al., 1998). CHO cells constitute a useful model to investigate h5-HT1A receptor coupling because they express Gaq and Gaq2 (Gerhardt and Neubig, 1991; Law et al., 1993; Gettys et al., 1994b). CHO cells also express low levels of Gao, but Gai2 is undetectable (Gerhardt and Neubig, 1991; Gettys et al., 1994b; Law et al., 1993; present study). Using antibody capture/SPA methods, we show here that high-efficacy agonists, but not partial agonists or inverse agonists, exhibit bell-shaped isotherms for Gaq activation at h5-HT1A receptors. The data indicate that low concentrations of high-efficacy agonists direct coupling of the receptor to Gai3, whereas, at higher concentrations, this coupling is suppressed. A preliminary report of the present data was presented in abstract form (Newman-Tancredi et al., 2002).

Materials and Methods
Membranes Preparations from CHO-h5-HT1A cells. Membranes from transfected CHO cells stably expressing the human serotonin 5-HT1A (h5-HT1A) receptor (~4 pmol/mg) were prepared as described previously (Newman-Tancredi et al., 1998). Briefly, cells were grown in RPMI 1640 medium containing 10% (v/v) fetal bovine serum, penicillin, and streptomycin until they reached confluence. Cells were harvested by centrifugation and homogenized using a Polytron homogenizer (Kinematica, Basel, Switzerland) in buffer A (20 mM HEPES, pH 7.4, and 3 mM MgCl2). The homogenate was centrifuged at 50,000 g for 30 min, the membrane pellet resuspended in buffer A and stored at ~8°C. Protein concentration was determined by use of a bichinonic acid kit (Sigma-Aldrich, St. Quentin-Fallavier, France).

### Table 1

<table>
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<tr>
<th>Ligand</th>
<th>E_{min}</th>
<th>E_{max}</th>
<th>pEC_{50,eff}</th>
<th>pEC_{50,isol}</th>
<th>% High</th>
<th>n_{H}</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>Control</td>
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<td>9.09 ± 0.03</td>
<td>7.70 ± 0.05</td>
<td>59 ± 3</td>
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<td>87 ± 3</td>
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<tr>
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<td>221 ± 16</td>
<td>9.16 ± 0.04</td>
<td>7.81 ± 0.16</td>
<td>55 ± 2</td>
<td>0.69 ± 0.05</td>
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<tr>
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<td>7.88 ± 0.05</td>
<td>7.79 ± 0.32</td>
<td>101 ± 3</td>
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<tr>
<td>+ anti-Ga1,2</td>
<td>82 ± 2</td>
<td>101 ± 3</td>
<td>7.93 ± 0.32</td>
<td>7.93 ± 0.32</td>
<td>101 ± 3</td>
<td>0.98 ± 0.08</td>
<td>3</td>
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<tr>
<td>+ anti-pERK</td>
<td>97 ± 2</td>
<td>133 ± 2</td>
<td>8.18 ± 0.17</td>
<td>8.18 ± 0.17</td>
<td>133 ± 2</td>
<td>0.78 ± 0.08</td>
<td>3</td>
</tr>
</tbody>
</table>

*pEC_{50,isol} value (single site fit).*

Membranes were preincubated either with buffer (control conditions) or with an antibody against Ga1,2 subunits or against pERK. The full agonist 5-HT or the partial agonist pindolol and [35S]GTPγS were then added and incubated for a further 1 h before filtering the membranes and scintillation counting. Isotherms were analyzed by nonlinear regression. E_{max} values are the peak signal (35S)GTPγS binding observed in the absence of ligand, and are expressed as a percentage of control basal values (100%). E_{min} values are the maximal observed stimulation (relative to control basal values = 100%). pEC_{50,eff} and pEC_{50,isol} are effective concentration values for the high and low potency components. % High are the percentage of sites in the high-potency component, and n_{H} is the slope of the isotherms. Data are expressed as mean ± S.E.M. of n independent determinations performed in triplicate. In the case of 5-HT, isotherms were biphasic (P < 0.05, F-test), except when preincubated with anti-Ga1,2. Pindolol yielded monophasic isotherms in all cases. Anti-Ga1,2, but not anti-pERK, antibodies reduced basal [35S]GTPγS binding from control values.
[35S]GTPγS Binding by Filtration Assays. Unless stated otherwise, [35S]GTPγS binding detecting “total” G-protein activation (i.e., without distinguishing between G-protein subtypes) was performed essentially as described previously (Newman-Tancredi et al., 1997, 1998). Briefly, CHO-h5-HT1A membranes (~25 μg) were incubated (60 min, 22°C) in duplicate in a buffer containing 20 mM HEPES, pH 7.4, 3 μM GDP, 3 mM MgCl2, 100 mM NaCl and 0.2 nM [35S]GTPγS (1300 Ci/mmol; PerkinElmer Life Sciences, Boston, MA). Nonspecific binding was defined with 10 μM GTPγS. Agonist efficacy is expressed relative to basal (100%). In antagonist tests, the antagonist was preincubated with cell membranes (30 min, 22°C) before addition of [35S]GTPγS. Experiments were terminated by rapid filtration through Whatman GF/B filters using a Packard (PerkinElmer) cell harvester and radioactivity determined by liquid scintillation counting.

[35S]GTPγS Binding by Antibody Capture and Scintillation Proximity Assay Detection. To specifically detect [35S]GTPγS binding to Goi3 G-protein subunits, an antibody-capture strategy was adopted, coupled to detection by scintillation proximity assay (SPA). Procedures were similar to those described by DeLapp et al. (1999). Unless otherwise indicated, CHO-h5-HT1A cell membranes (~35 μg) were incubated on 96-well plates with agonists and/or antagonists and 0.2 nM [35S]GTPγS for 1 h at 22°C in a buffer containing: 20 mM HEPES, pH 7.4, 3 μM GDP, 3 mM MgCl2, and 100 mM NaCl (i.e., the same buffer composition as for filtration assays described above). Reaction was stopped by solubilizing cell membranes by addition of detergent [Nonidet P40, 0.3% (v/v) final] and gentle agitation for 30 min. Mouse anti-Goi1/3 monoclonal anti-

Fig. 1. Disruption of receptor/G-protein interaction in CHO-h5-HT1A cell membranes by anti-Goi1/3 antibodies. CHO-h5-HT1A cell membranes were preincubated with anti-Goi1/3 monoclonal antibodies (0.2 μg/well) before carrying out [35S]GTPγS binding and filtering cell membranes. The presence of the antibody (but not an anti-pERK IgG) reduced constitutive Goi3 activation and blocked the high-affinity 5-HT stimulation component. Top, influence of antibodies on 5-HT-stimulated [35S]GTPγS binding. Anti-Goi1/3 antibodies blocked high-affinity stimulation and lowered basal binding. Middle, the same isomers from A were normalized to show the loss of the high-affinity stimulation component. Bottom, influence of antibodies on pindolol-stimulated [35S]GTPγS binding. Anti-Goi1/3 antibodies suppressed stimulation without a change in potency. Data points are means of triplicate determinations from representative experiments repeated on at least three independent occasions. pEC50 and Emax values from these experiments are shown in Table 1.

Fig. 2. Bell-shaped isotherms of G-protein activation in CHO-h5-HT1A cell membranes. [35S]GTPγS Binding assays were carried out by classical filtration methods. Top, 5-HT stimulated [35S]GTPγS binding in a biphasic manner (P < 0.01, F-test). Addition of unlabeled GTPγS at the end of the incubation period time-dependently suppressed [35S]GTPγS binding at high concentrations of 5-HT but not at 30 nM 5-HT. Bottom, pindolol acted as a partial agonist. Addition of unlabeled GTPγS at the end of the incubation period time-dependently suppressed [35S]GTPγS binding uniformly at all pindolol concentrations. Data points are means of triplicate determinations from representative experiments repeated on at least three independent occasions with similar results.
bodies (Biomol, Plymouth Meeting, PA) were then added (0.1 μg of IgG per well) and the microwell plates incubated for a further 1 h to allow antibody-Go complexes to form. Because CHO cells do not express Go1a (Gettys et al., 1994b; see Introduction), the assay detects activation of Go1i. The specificity of the anti-Go1i antibody itself was verified by Western blots against a range of purified Go subunits, indicating an absence of cross-reactivity with Go2i, Go3i, Go5i, Go7i, and Go13i (Newman-Tancredi et al., 2002). At the end of the incubation period, SPA beads coated with anti-mouse second antibody (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK), were added at the manufacturer’s recommended concentrations and incubated with gentle agitation overnight before radioactivity counting. All incubation steps were carried out at 22°C. Nonspecific binding was defined with 10 μM GTP. Results are expressed as the mean ± S.E.M. of n determinations. A monoclonal antibody against phosphorylated extracellular signal regulated kinases (pERK; Nanotools, Germany) was employed to exclude nonspecific effects.

Data Analysis. [35S]GTPγS binding data were analyzed by nonlinear regression using the program Prism (GraphPad Software Inc., San Diego, CA). For conditions that produced sigmoidal isotherms, values of potency (pEC50) and pseudo-Hill coefficient (nH) were determined. The maximal observed stimulation (Emax) was defined as the amount of specific [35S]GTPγS binding expressed as a percentage of specific basal (agonist-independent) binding (100%). The “goodness of fit” was tested by “runs” test, and one- and two-site fits were compared by F-test. In cases where a two-site fit was more favorable, potency values are shown for the high- (pEC50H) and low-affinity (pEC50L) binding components.

Fig. 3. Dissociation of [35S]GTPγS from CHO-h5-HT1A cell membranes. Top, CHO-h5-HT1A cell membranes were preincubated for 60 min with [35S]GTPγS and 5-HT (30 nM) or 5-HT (10 μM) or no ligand (basal conditions). Unlabeled GTPγS was then added, resulting in a more rapid decrease of binding with 10 nM 5-HT (to basal levels) than with 100 nM 5-HT. Bottom, CHO-h5-HT1A cell membranes were preincubated for 60 min with [35S]GTPγS and pindolol (10 μM) or spiperone (10 μM). When unlabeled GTPγS was added, the decrease in binding paralleled that under basal conditions. Data points are means of triplicate determinations from representative experiments repeated on at least three independent occasions. t1/2 values from these experiments are described in the text.

Fig. 4. Bell-shaped and sigmoidal isotherms: parameters analyzed. Top, a theoretical bell-shaped isotherm is shown to illustrate the various parameters analyzed. a1 = [35S]GTPγS binding observed in the absence of ligand (basal conditions). a2 = [35S]GTPγS binding observed at maximal ligand concentration. c1 = drug concentration inducing half-maximal stimulation. c2 = drug concentration inducing half of the inhibitory effect. Emax = maximal stimulation induced by agonists and maximal inhibition induced by inverse agonists.
For agonist activation of G_{i3} yielding bell-shaped curves, iso-
therms were analyzed using by a four-parameter logistic equation.
The ascending (data points up to maximal stimulation) and descend-
ing (data points from maximal stimulation onwards) components of
the isotherms were analyzed independently employing a four-param-
eter logistic equation: \( [^{35}S]\text{GTP-S bound} = a_1 + b_1 \times \exp (-c_1 \times x + b_1 \times x^2) \), where \( a_1 = [^{35}S]\text{GTP-S binding observed in the absence of ligand (basal conditions), } a_2 = [^{35}S]\text{GTP-S binding observed at maximal ligand concentration, } b_1 = \text{ Hill slope of the ascending component of the isotherms, } b_2 = \text{ Hill slope of the descending component of the isotherms, } c_1 = \text{ drug concentration inducing half-maximal stimulation, } c_2 = \text{ drug concentration inducing half of the inhibitory effect, } d = \text{ maximal stimulation of } [^{35}S]\text{GTP-S binding} \) (Rovati and Nicosia, 1994). Fig. 4 illustrates the various parameters analyzed.

Antagonist potency (pot) values for dendrit shift of bell-shaped
isotherms of agonist-stimulated G_{i3} activation was calculated by:
\( \text{pot} = \frac{[\text{Antagonist}]/[\text{EC}_{[50]}]}{[\text{Antagonist}]/[\text{EC}_{[50]}] - 1}, \) where [Antagonist] = antagon-
tagonist concentration; [EC_{[50]}] = concentration of agonist producing half-maximal stimulation/inhibition; and [EC_{[50]}] = concentration of agonist producing half-maximal stimulation/inhibition in the presence of antagonist. Antagonist potency for reversal of pindolol-stimu-
lated or spiperone-inhibited G_{i3} activation were calculated by:
\( \text{pot} = \frac{[\text{Ligand}]/[\text{EC}_{[50]}]}{[\text{Ligand}]/[\text{EC}_{[50]}] - 1} \); where Ligand = pindolol or spiperone concentration; and \( n_H \) = Hill coefficient of pindolol or spiperone alone.

Raw data from individual experiments performed in duplicate or
triplicate were analyzed independently to yield the relevant param-
eters (pEC_{50}, R_max, etc.). Data in tables are expressed as the mean ±
S.E.M. values from at least three independent experiments. For illustrative purposes, figures show isotherms from representative experiments; data points on graphs are means of duplicate or triplicate determi-
inations.

**Drugs.** Clozapine base, (-)-pindolol base, (+)-8-OH-DPAT HBr and 4-ido-N-(2-[4-(methoxyphenyl)-1-piperazinyl][ethyl]-N-2-pyridi-
nyl-benzamide HCl (p-MPPI), (+)-butaclamol HCl, spiperone HCl, and (+)-5-fluro-8-hydroxy-dipropylaminotetralin HCl were ob-
tained from Sigma/RBI (Natick, MA); 5-HT creatinine sulfate, halo-
peridol base, and buspirone HCl were obtained from Sigma-Aldrich;
5-HT and 4-ido-N-[2-[4-(methoxyphenyl)-1-piperazinyl][ethyl]-N-
2-pyridinyl-benzamide HCl (p-MPPI), (+)-butaclamol HCl, spiperone HCl, and (+)-5-fluro-8-hydroxy-dipropylaminotetralin HCl were ob-
tained from Sigma/RBI (Natick, MA); 5-HT creatinine sulfate, halo-
peridol base, and buspirone HCl were obtained from Sigma-Aldrich;
S15535 base, eltoprazine HCl, and WAY100,635 were synthesized by
J. L. Peglion, Servier (Suresnes, France). 5-Carboxyamidot-
rutamycin (5-CT) maleate, S16924, S14506, S14671, (-)-flesinoxan
HCl, and ziprasidone HCl were synthesized by G. Lavielle, Servier
(Croissy-sur-Seine, France). Methiothepin maleate was from Tocris
Cookson (Southampton, England).

### Results

**Total G-Protein Activation Determined by Filtration Experiments**

**Disruption of h5-HT_{1A} receptor-G-protein interactions with anti-G_{i3} antibody.** 5-HT concentration-de-
dependently stimulated [^{35}S]GTP-S binding to CHO-h5-HT_{1A}
cell membranes by ~2.2-fold relative to basal values (Table 1).
The slope factors (pseudo-Hill coefficients) were less than unity (0.72) and a two-site fit was significantly superior to a single-site fit (P < 0.01; F-test) yielding a pEC_{50} of 9.09 and a pEC_{50} of 7.70 (Table 1). We have previously shown that 5-HT-stimulated [^{35}S]GTP-S binding is totally reversed by the selective 5-HT_{1A} receptor antagonist, WAY100,635 (New-
man-Tancredi et al., 1996). When CHO-h5-HT_{1A} cell mem-
branes were preincubated (2 h, 22°C) with an anti-G_{i3} mono-
clonal antibody (0.2 μg/ml), the high potency stimulation
component was abolished, yielding a monophasic iso-
therm: pEC_{50} = 8.15 (Fig. 1B). Further, basal binding was
reduced relative to control basal values, suggesting that con-
stitutive G_{i3} activation was attenuated. The partial agonist,
pindolol, yielded monophasic stimulation isotherms (Table 1;
\( \text{pEC}_{50} = 7.88 \)). Anti-G_{i3/3} preincubation did not modify the potency of pindolol (pEC_{50} = 7.79) but markedly reduced its
efficacy (Fig. 1C) suggesting that pindolol preferentially in-
duces h5-HT_{1A} coupling to G_{i3} subunits. A monoclonal
antibody against pERK did not markedly modify 5-HT- or pin-
dolol-induced [^{35}S]GTP-S binding (Fig. 1, A and C).

**Influence of 5-HT and Pindolol on Dissociation Kinetics of [^{35}S]GTP-S.** We investigated the rates of GTP/
GDP exchange at G-proteins in CHO-h5-HT_{1A} membranes by preincubating them with [^{35}S]GTP-S and 5-HT (1 h, 22°C)
and then adding an excess of unlabeled GTP-S (10 μM final
concentration). [^{35}S]GTP-S binding was time-dependently in-
hibited at high concentrations (e.g., 10 μM) but not at lower
concentrations (e.g., 30 nM) of 5-HT, which stimulated G_{i3}

### Table 2

Bell-shaped isotherms for h5-HT_{1A} receptor-mediated stimulation of [^{35}S]GTPγS binding to G_{i3} subunits

<table>
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<tr>
<th>Ligand</th>
<th>( c_1 )</th>
<th>( d )</th>
<th>( c_2 )</th>
<th>( a_2 )</th>
<th>( n )</th>
<th>( \text{pK}_i )</th>
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<td>5-HT</td>
<td>9.22 ± 0.04</td>
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<td>7.31 ± 0.06</td>
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<td>S14506</td>
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<td>Clozapine</td>
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<td>82 ± 6</td>
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<td>5-CT</td>
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<tr>
<td>(+)-7-OH-DPAT</td>
<td>7.02 ± 0.06</td>
<td>267 ± 19</td>
<td>5.17 ± 0.09</td>
<td>129 ± 16</td>
<td>3</td>
<td>7.29</td>
</tr>
<tr>
<td>Eltoprazine</td>
<td>8.14 ± 0.01</td>
<td>232 ± 9</td>
<td>6.34 ± 0.02</td>
<td>143 ± 5</td>
<td>3</td>
<td>8.19</td>
</tr>
<tr>
<td>Buspirone</td>
<td>8.23 ± 0.07</td>
<td>234 ± 2</td>
<td>6.45 ± 0.03</td>
<td>153 ± 3</td>
<td>3</td>
<td>8.05</td>
</tr>
<tr>
<td>S16924</td>
<td>8.67 ± 0.06</td>
<td>236 ± 13</td>
<td>6.94 ± 0.05</td>
<td>166 ± 16</td>
<td>3</td>
<td>8.74</td>
</tr>
<tr>
<td>(+)-8-OH-DPAT</td>
<td>9.09 ± 0.08</td>
<td>284 ± 20</td>
<td>7.27 ± 0.16</td>
<td>220 ± 20</td>
<td>3</td>
<td>9.19</td>
</tr>
</tbody>
</table>

\( a_2 \) = maximal inhibition of [^{35}S]GTP-S binding; \( c_1 \) = drug concentration inducing half-maximal stimulation; \( c_2 \) = drug concentration inducing half of the inhibitory effect; \( d \) = maximal observed stimulation relative to basal values (×100%).
(see above). Thus, after 40 min of incubation, a bell-shaped isotherm was observed, with a peak at about 30 nM of 5-HT (Fig. 2A). In comparison, pindolol-induced [35S]GTPγS binding (Fig. 2B) was uniformly and progressively diminished by incubation with GTPγS (Emax after 40 min with GTPγS = 121 ± 3%). No bell-shaped isotherms appeared and the potency of pindolol was unchanged (pEC50 after 40 min with GTPγS = 7.85 ± 0.20). The dissociation kinetics of [35S]GTPγS were directly assessed by adding GTPγS (10 μM) after the standard 1-h incubation period and determining the remaining bound [35S]GTPγS after different dissociation times (Fig. 3). [35S]GTPγS binding in the presence of 10 μM 5-HT decreased to basal levels within 60 min (t1/2 = 6.16 ± 0.69 min for a single-site model). Comparison of single- and two-site fits for 10 μM 5-HT revealed that its dissociation isotherm was biphasic (first component, t1/2 = 2.4 ± 1.2 min, 51 ± 8% of binding sites; second component, t1/2 = 24.6 ± 7.0 min; p < 0.05 in F-test) suggesting that [35S]GTPγS was dissociating from 2 G-protein populations. In contrast, dissociation isotherms with 30 nM 5-HT or 10 μM pindolol (which preferentially induce coupling to Gαi3) decreased monophasically and more slowly (t1/2 = 21.53 ± 3.05 and 8.99 ± 0.48 min, respectively; Fig. 3).

**Gαi3 Subunit Activation**

The antibody capture/SPA technique was adopted to specifically detect Gαi3 subunit activation. The agonist, 5-HT, yielded bell-shaped [35S]GTPγS binding isotherms (see Fig. 4 for analysis parameters), with peak stimulation at a concentration of about 5 nM (Table 2). [35S]GTPγS binding then gradually diminished to below baseline values at 5-HT concentrations of about 1 μM (Fig. 5). A series of experiments was carried out to verify the specificity of Gαi3 detection by comparing the influence of the full agonist 5-HT with that of an efficacious inverse agonist, spiperone (Newman-Tancredi et al., 1997). First, in Gαi3/SPA assays, 5-HT and spiperone did not modify [35S]GTPγS binding to membranes of untransfected CHO cells (Fig. 5A). Second, when experiments were carried out in the absence of anti-Gαi3 antibodies or in the presence of another IgG (monoclonal anti-pERK), no stimulation of [35S]GTPγS binding was detected, although a slight loss of signal was observed at high 5-HT concentrations in some experiments (Fig. 5B). Third, we tested SPA beads coated with anti-rabbit IgG (as opposed to anti-mouse IgG). Once again, no stimulation of [35S]GTPγS binding was detected (data not shown). Fourth, we pretreated CHO-h5-HT1A cells overnight with the ADP-ribosylating agent PTX (100 ng/ml). 5-HT failed to induce Gαi3 stimulation in membranes prepared from these cells. Similarly, no inhibition of [35S]GTPγS binding by the inverse agonist, spiperone, was detected in PTX-pretreated cell membranes (Fig. 5C). Fifth, we tested SPA beads coated with wheat-germ agglutinin instead of IgG. Wheat-germ agglutinin SPA beads bind cell membranes without distinguishing G-protein subtypes. As expected, the resulting isotherms for 5-HT, (+)-8-OH-DPAT, pindolol, and spiperone closely resembled (both in potency and efficacy) those observed in parallel standard filtration assays (data not shown). Sixth, we verified the absence of Gαi3 subunits in the present CHO cell membranes. A specific polyclonal anti-Gαi3 antibody (I20; Santa Cruz Biotechnology, Santa Cruz, CA) detected recombinant purified Gαi3 subunits in Western blot experiments but did not detect a band in cell membranes from CHO-h5-HT1A cells (data not shown), in agreement with reports from other laboratories (Gettys et al., 1994b; see Introduction).

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**Figure 5.** [35S]GTPγS binding to Gαi3 subunits: G-protein and receptor specificity. H5-HT1A receptor-mediated Gαi3 subunit activation was determined by antibody capture/SPA detection. A, detection of 5-HT-stimulated and spiperone-inhibited [35S]GTPγS binding to Gαi3 subunits in CHO-h5-HT1A cell membranes but not in membranes of untransfected CHO cells. B, detection of 5-HT-stimulated [35S]GTPγS binding to Gαi3 subunits in CHO-h5-HT1A cell membranes employing a specific monoclonal anti-Gαi3 antibody. No stimulation was observed when an anti-MAPK monoclonal or no antibody are used. C, CHO-h5-HT1A cells were pre-treated overnight with pertussis toxin (50 ng/ml) before preparing cell membranes. 5-HT-stimulated and spiperone-inhibited [35S]GTPγS binding to Gαi3 subunits was abolished in PTX-treated but not control CHO-h5-HT1A cell membranes. Data points are means of duplicate determinations from representative experiments repeated on at least three independent occasions with similar results.
Modulation of Go13 Activation by Buffer/Incubation Components

We varied the standard incubation buffer (3 μM GDP, 3 mM MgCl2, and 100 mM NaCl) to examine the influence of buffer components on Go13 subunit activation by the full agonist (5-HT), a partial agonist (pindolol), and an inverse agonist (spiperone). Each parameter was varied individually while the others were maintained at standard concentrations.

**Influence of GDP (0.03, 0.3, 3.0, and 30 μM).** GDP concentration influences the rate of GTP/GDP exchange by Go subunits. As [GDP] increased, basal binding was suppressed such that, at 30 μM GDP, basal binding was only slightly greater than nonspecific binding determined in the presence of 10 μM GTPγS. Under these conditions, very little stimulation of [35S]GTPγS binding was induced by 5-HT or pindolol (Fig. 6, A and B). 5-HT yielded bell-shaped isotherms and pindolol yielded sigmoidal isotherms in all cases, but stimulation was greatest when [GDP] = 3 μM. In contrast, spiperone yielded the greatest decrease in dpm at the lowest GDP concentrations [i.e., when high constitutive activity was favored (Fig. 6C)].

**Influence of MgCl2 (0.06, 0.33, 3.0, and 30 mM).** Mg2+ ions are cofactors for GTP binding to G-proteins. Thus, at low [MgCl2], basal [35S]GTPγS binding was only slightly higher than nonspecific binding. As [MgCl2] increased, basal binding increased but the concentration-response isotherms of 5-HT remained bell-shaped in all cases (Fig. 6D). The peaks shifted to the left as [MgCl2] increased to 3 mM and then to the right at 30 mM, suggesting that agonist potency (and not just efficacy) is dependent on an optimal Mg2+ concentration. Stimulation by pindolol and inhibition by spiperone were most accentuated at 3 mM MgCl2 (Fig. 6, E and F).

**Influence of Sodium Chloride (10, 30, 100, and 300 mM).** Monovalent cations, such as Na+, modulate receptor interactions with G-proteins and the concentration of NaCl was the only parameter to fundamentally modify the shape of the Go13 activation isotherms. Thus, at low [NaCl], high basal binding was observed and no stimulation by 5-HT: the only action of 5-HT was to inhibit Go13 activation (Fig. 6G). Conversely, at high [NaCl], the stimulatory actions of 5-HT were evident, but not the inhibitory actions. Pindolol stimulated Go13 activation at 100 mM NaCl but inhibited it at 10 mM NaCl (Fig. 6H). Spiperone displayed the greatest inhibi-

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![Fig. 6](image_url)  
**Fig. 6.** Influence of ionic conditions and guanine nucleotides on [35S]GTPγS binding to Go13 subunits in CHO-h5-HT1A cell membranes. h5-HT1A receptor-mediated Go13 subunit activation was determined by antibody capture/SPA detection. The influence of four concentrations each of GDP (A, B, and C), MgCl2 (D, E, and F), and NaCl (G, H, and I) on the actions of a full agonist (5-HT), a partial agonist (pindolol), and an inverse agonist (spiperone) are shown. Data points are means of duplicate determinations from representative experiments repeated on at least three independent occasions with similar results.
tion of G\(_{\alpha_{i3}}\) activation at low [NaCl]—where basal binding was highest.

**Influence of Incubation Time (5, 10, 20, and 60 min).**
The appearance of bell-shaped G\(_{\alpha_{i3}}\) activation isotherms for 5-HT was time-dependent. At short incubation times, isotherms were essentially sigmoidal, whereas at longer incubation times, bell-shaped isotherms were observed due to stimulation of G\(_{\alpha_{i3}}\) in the presence of nanomolar, but not higher, concentrations of 5-HT (Fig. 7).

**Influence of Agonists, Antagonists, and Inverse Agonists**

As well as 5-HT (described above), other full agonists (as defined by classical filtration assays, Newman-Tancredi et al., 1998, 2001a,b), including (\(+\)8-OH-DPAT, flesinoxan, and S14506, yielded bell-shaped \[^{35}\text{S}\]GTP\(^{\gamma}\)S binding isotherms, with peak stimulation at drug concentrations shown in Table 2. Like 5-HT, high concentrations of these ligands reduced \[^{35}\text{S}\]GTP\(^{\gamma}\)S binding below basal values (Fig. 8). The partial agonists buspirone and S16924 (Newman-Tancredi et al., 2001b), yielded broader bell-shaped isotherms which did not return to baseline values. The isomers (+-8-OH-DPAT and (\(-\)8-OH-DPAT differed dramatically; the former yielded bell-shaped isotherms but the latter did not (Fig. 8). Two other partial agonists, S15535 and pindolol (Newman-Tancredi et al., 1996, 2001a,b), also induced sigmoidal isotherms. The neutral antagonist WAY100,635 and another antagonist, p-MPPI, modestly stimulated \[^{35}\text{S}\]GTP\(^{\gamma}\)S binding with sigmoidal concentration-response curves (\(E_{\text{max}}\) = 135 and 128%, respectively; Table 3), indicating mild agonist properties for activation of G\(_{\alpha_{i3}}\). Sigmoidal binding curves were also observed with the inverse agonists, spiperone, haloperidol, (+)-butaclamol and methiothepin, which inhibited basal \[^{35}\text{S}\]GTP\(^{\gamma}\)S binding, consistent with constitutive activation of G\(_{\alpha_{i3}}\) (Table 3).

**Antagonist Studies.** Two kinds of experiments were carried out with the selective 5-HT\(_{1A}\) receptor antagonist, WAY100,635. i) concentration-response isotherms of 5-HT,
(±)-OH-DPAT, pindolol, and spiperone were shifted in parallel to the right by fixed concentrations (3 or 10 nM) of WAY100,635 without loss of maximal stimulation (Fig. 9), consistent with competitive antagonist actions. \( pK_i \) values (shown in Table 4) were similar for both the ascending and descending components of the bell-shape isotherms suggesting that they are mediated by a single binding site. ii) Pindolol (100 nM)-induced stimulation and spiperone (1 \( \mu \)M)-induced inhibition of \( G_{\alpha_i} \) activation were concentration-dependently reversed by WAY100,635. \( pK_i \) values resembled the \( pK_i \) (9.91) of WAY100,635 at h5-HT\(_{1A}\) receptors (Newman-Tancredi et al., 2001a).

**Discussion**

**Total G-Protein Activation Assays.** In studies of total G-protein activation, which do not distinguish between G-protein subtypes, 5-HT stimulated \([^{35}S]\)GTP\(\gamma\)S binding biphasically, suggesting the presence of multiple h5-HT\(_{1A}\) receptor/G-protein coupling conformations. Indeed, preincubation of CHO-h5-HT\(_{1A}\) cell membranes with an antibody that recognizes \( G_{\alpha_i} \) subunits selectively abolished the high potency 5-HT stimulation component and decreased basal binding (Fig. 1, A and B). Although the G-protein activated at high concentrations of 5-HT remains to be formally identified, it is probably \( G_{\alpha_i} \), a subtype highly expressed in CHO cells and known to interact with h5-HT\(_{1A}\) receptors (see Introduction). Indeed, in preliminary antibody-capture experiments, 5-HT stimulated \([^{35}S]\)GTP\(\gamma\)S binding to \( G_{\alpha_i} \) (as opposed to \( G_{\alpha_l} \) described below) with a \( pEC_{50} \) of 7.3 (A. Newman-Tancredi and M. J. Milian; unpublished observations), similar to the low potency component observed in filtration experiments (\( pEC_{50} \) 7.7; Table 1). The involvement of multiple G-protein populations in receptor coupling can be shown by investigation of their contrasting GTP/GDP exchange kinetics (Shea et al., 2000). Indeed, Wenzel-Seifert and Seifert (2000) have shown that Gs, Gaq, and \( G_{\alpha_l} \) differ in their rates of \([^{35}S]\)GTP\(\gamma\)S dissociation. Herein, when CHO-h5-HT\(_{1A}\) cell membranes were exposed to unlabeled GTP\(\gamma\)S after the standard incubation period, nanomolar concentrations of 5-HT (or saturating concentrations of the partial agonist, pindolol) stabilized \([^{35}S]\)GTP\(\gamma\)S binding and slowed its dissociation from CHO-h5-HT\(_{1A}\) cell membranes, whereas dissociation was accelerated by micromolar concentrations of 5-HT (Fig. 3). Taken together, these experiments indicate that pindolol or low concentrations of 5-HT preferentially induce coupling of h5-HT\(_{1A}\) receptors to \( G_{\alpha_i} \) subunits, but that coupling to this G-protein is suppressed at high concentrations of 5-HT, revealing bell-shaped concentration-response isotherms. These observations are reminiscent of a study by van Hooft and Vijverberg (1996) on 5-HT\(_{3}\) receptors and strongly suggest that full and partial agonists select distinct conformational states of h5-HT\(_{1A}\) receptors.

**Bell-Shaped Isotherms for \( G_{\alpha_i} \) Activation: Specific Mediation by 5-HT\(_{1A}\) Receptors.** To directly investigate h5-HT\(_{1A}\) receptor coupling to \( G_{\alpha_i} \), we employed an antibody capture/SPA technique similar to that described previously for muscarinic receptors (DeLapp et al., 1999). Initial experiments revealed that 5-HT induced a concentration-dependent rise and then fall in \([^{35}S]\)GTP\(\gamma\)S labeling of \( G_{\alpha_i} \) subunits, with a striking resemblance to the isotherms obtained from filtration assays after incubation with GTP\(\gamma\)S (Fig. 2). As discussed previously (Szabadi, 1977; Rovati and Nicosia, 1994; Tucek et al., 2001), several mechanisms may give rise to bell-shaped isotherms in pharmacological systems. First, different signal transduction systems may be activated by ligand binding at two independent receptors (for examples, see Okpako, 1972; Lejeune et al., 1997). In the present system, however, stimulation of \( G_{\alpha_i} \) subunits is entirely attributable to activation via h5-HT\(_{1A}\) receptors. Indeed, there was no influence of 5-HT on \( G_{\alpha_i} \) activation in nontransfected CHO cells and the entire bell-shaped isotherms of 5-HT and the selective 5-HT\(_{1A}\) receptor agonist (+)-8-OH-DPAT were right-shifted by the selective antagonist WAY100,635 (Table 4; Fletcher et al., 1996; Newman-Tancredi et al., 1997). Hence, the presence of multiple receptor types can be ruled out, although the possibility remains that agonists may be sequentially acting at multiple binding sites on target receptors, as has been proposed for bell-shaped mobilization of calcium ions by muscarinic agonists in SH-SY5Y neuroblastoma cells (Järvi et al., 1995). However, that study did not report whether agonist actions could be blocked by antagonists. On the other hand, a preliminary study by Browning et al. (2000a) showed bell-shaped isotherms for adenosine A\(_1\) receptor-mediated \([^{35}S]\)GTP\(\gamma\)S binding in CHO cell membranes that were blocked by a selective antagonist (8-cyclo-

**TABLE 3**

Sigmoidal isotherms for h5-HT\(_{1A}\) receptor-mediated stimulation of \([^{35}S]\)GTP\(\gamma\)S binding to \( G_{\alpha_i} \) subunits

<table>
<thead>
<tr>
<th>Ligand</th>
<th>( pEC_{50}/pIC_{50} )</th>
<th>( E_{\max}/E_{\min} )</th>
<th>( n )</th>
<th>( pK_i )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agonists</td>
<td></td>
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<td></td>
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<tr>
<td>S15535</td>
<td>8.91 ± 0.06</td>
<td>234 ± 16</td>
<td>3</td>
<td>9.10</td>
</tr>
<tr>
<td>(--)Pindolol</td>
<td>8.24 ± 0.03</td>
<td>193 ± 10</td>
<td>5</td>
<td>8.19</td>
</tr>
<tr>
<td>WAY100,635</td>
<td>8.52 ± 0.06</td>
<td>135 ± 5</td>
<td>3</td>
<td>9.25</td>
</tr>
<tr>
<td>p-MPPI</td>
<td>8.32 ± 0.07</td>
<td>128 ± 5</td>
<td>3</td>
<td>9.00</td>
</tr>
<tr>
<td>(--)UH301</td>
<td>8.24 ± 0.23  ( a )</td>
<td>111 ± 1  ( a )</td>
<td>2</td>
<td>7.87</td>
</tr>
<tr>
<td>Inverse agonists</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methiothepin</td>
<td>7.84 ± 0.18</td>
<td>80 ± 2</td>
<td>3</td>
<td>8.08</td>
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<tr>
<td>(--)Butaclamol</td>
<td>6.47 ± 0.22</td>
<td>72 ± 3</td>
<td>3</td>
<td>6.40</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>5.55 ± 0.14</td>
<td>69 ± 18</td>
<td>3</td>
<td>5.72</td>
</tr>
<tr>
<td>Spiperone</td>
<td>7.33 ± 0.07</td>
<td>27 ± 3</td>
<td>7</td>
<td>7.00</td>
</tr>
</tbody>
</table>

\( pEC_{50} \) agonist concentration inducing half-maximal stimulation; \( pIC_{50} \) inverse agonist concentration inducing half of the inhibitory effect; \( E_{\max} \) maximal observed agonist stimulation relative to specific basal binding (100%); \( E_{\min} \) maximal inverse agonist inhibition of \([^{35}S]\)GTP\(\gamma\)S binding relative to specific basal binding (100%).

\( a \) Mean ± range.
pentyl-1,3-dipropylxanthine), although the precise G-protein subtypes involved were not identified.

A second situation that can give rise to bell-shaped isotherms is when an agonist-activated receptor couples to multiple G-proteins that mediate opposing actions on a downstream effector. Thus, at porcine α2A adrenoceptors, the agonist UK14,304, can both inhibit adenyl cyclase (via PTX-sensitive Gi/o G-proteins) and stimulate it (presumably via Gs; Brink et al., 2000). Similarly, cannabinoid CB1 and muscarinic m2 receptors can both inhibit and (less potently) stimulate cAMP accumulation. In PTX-pretreated cells, only the stimulatory actions of agonists on cAMP accumulation are observed, an effect attributed to coupling to Gs (Bonhaus et al., 1998; Tucek et al., 2001). A recent study reported similar effects for h5-HT1A receptors (Malmberg and Strange, 2000). In contrast to these studies, in the present system both agonist and inverse agonist modulation of [35S]GTPγS binding were abolished by PTX, indicating that only Gi/o G-proteins are involved in their actions (Fig. 5). Further, the specificity of Goαi3 activation was shown by the fact that no stimulation was detected in the absence of antibody or when another antibody (anti-pERK) was used. Hence, the data support the conclusion that the bell-shaped isotherms are specifically due to activation by h5-HT1A receptors in CHO cell membranes of a single G-protein subtype (Goαi3).

Influence of Agonists, Partial Agonists, and Inverse Agonists for Goαi3 Activation. We investigated the activation of Goαi3 subunits by 22 drugs displaying diverse efficacies at 5-HT1A receptors (Tables 2 and 3). These ligands, which have been previously characterized by binding affinity ratios and classical [35S]GTPγS binding (Newman-Tancredi et al., 1996, 1998, 2001a,b), yielded widely differing Goαi3 activation patterns. First, spiperone, methiothepin, and the neuroleptics haloperidol and (−)-butaclamol exhibited inverse agonist properties (Figs. 8 and 9), in agreement with previous reports (Newman-Tancredi et al., 1998, 2001a,b; Cosi and Koek, 2001). Second, agonists that display high efficacy in classical filtration experiments (Newman-Tancredi et al., 1998), including 5-CT, (−)-flesinoxan, and S14506, exhibited bell-shaped isotherms, generally with narrow peaks. Third, weak partial agonist ligands, including S15535 and pindolol (Newman-Tancredi et al., 1996, 2001a), as well as p-MPPI and WAY100,635, yielded isotherms that were sigmoidal (Fig. 9). In the case of WAY100,635, this ligand is variously reported to behave as a ‘neutral antagonist’ in vivo and at CHO-h5-HT1A receptors (Fletcher et al., 1996; Newman-Tancredi et al., 1997), or as an inverse agonist at h5-HT1A receptors expressed in HeLa cells (Cosi and Koek, 2000). Further, a very recent GTPase activity study in human embryonic kidney 293 cells also detected weak partial agonist properties of WAY100,635 for activation of a h5-HT1A-GO1 fusion construct in the presence of co-expressed regulators of G-protein signaling-1 (RGS1; Welsby et al., 2002). Thus, WAY100,635 may have differential efficacy at different G-protein subtypes, behaving as a weak partial agonist at Goαi3 but as an antagonist or inverse agonist at other Goα subunits or under the influence of RGS proteins.

Taken together, the present data indicate that the behavior of partial agonists is qualitatively different from that of the full agonists. This is illustrated by the isomers of 8-OH-DPAT (Lejeune et al., 1997): whereas at low concentrations
(<3 nM) their stimulation curves were almost superimposable, at high concentrations, the -isomer returned to baseline (or below) but the -isomer did not. The present data differ from those of Gettys et al. (1994a) who investigated h5-HT1A receptor-mediated Gαi3 and Gαi5 activation in CHO cells using the photoreactive GTP analog 4-azidoanilido-[α-32P]GTP and reported sigmoidal isotherms for agonist activation of both these G-proteins. The absence of bell-shaped isotherms in their study may have been due to a lower receptor expression level: 0.9 pmol of h5-HT1A receptor/mg of protein for Gettys et al. (1994a) versus ~4 pmol/mg here. Indeed, high receptor expression may favor observation of bell-shaped isotherms (Browning et al., 2000a) and a preliminary study reported that bell-shaped isotherms for stimulation of [35S]GTPyS binding at adenosine A1 receptors were observed for high- but not low-efficacy agonists (Browning et al., 2000b).

Regulatory Mechanisms of Gαi3 Activation. Although the precise mechanisms involved in the bell-shaped concentration-response isotherms remains unclear, some tentative hypotheses may be proposed. First, a “strength-of-signal” mechanism may be involved in the present observations (Kenakin, 1995), whereby high-efficacy agonists stimulate multiple pathways, whereas lower efficacy agonists, such as S15535 and pindolol, can stimulate only the most efficiently-coupled pathway (in this case Gαi3). However, it is striking that S15535 and pindolol displayed maximal Gαi3 stimulation (Emax values 234 and 193%, respectively; Tables 2 and 3) comparable with that of 5-HT (245%), whereas they behaved as partial agonists in classical [35S]GTPyS binding experiments (Fig. 1 and Newman-Tancredi et al., 1996). Thus, S15535 and pindolol may be selectively trafficking agonist-directed signaling to Gαi3 subunits. This implies in turn that high efficacy for Gαi3 activation is not sufficient to induce the descending phase of bell-shaped isotherms. In fact, this downturn strongly suggests that when efficacious agonists attain a high level of occupation of h5-HT1A receptors, a conformational change may occur that induces coupling to other Gα subtypes and suppresses signaling through Gαi3 subunits. A similar concept has been evoked at muscarinic receptors (Dittman, et al. 1994). A change in receptor conformation is supported by the sensitivity of Gαi3 activation to the availability of Na+ and Mg2+ ions, as well as guanine nucleotides, which are known to influence h5-HT1A receptor-G-protein coupling (Fig. 6; Pauwels et al., 1997; Cosi and Koek, 2000). Indeed, a distinctive influence of NaCl on Gαi3 activation was observed: at high [NaCl], 5-HT stimulated Gαi3 activation sigmoidally (Fig. 6), but at low [NaCl], only inhibitory influences were detected. The presumed conformational change may involve formation of h5-HT1A receptor dimers, as has been reported for numerous GPCRs, including h5-HT1B and h5-HT1D receptors (Xie et al., 1999; Marshall, 2001). Such a hypothesis would account for the ability of high concentrations of high-efficacy agonists to decrease [35S]GTPyS binding below basal levels (Table 2; Fig. 9). Indeed, if dimers were no longer able to couple to Gαi3, constitutive activation of the latter would be suppressed by high concentrations of 5-HT, which would act, therefore, as a pseudo-inverse agonist. Receptor desensitization is another possible mechanism affecting receptor conformation, and its occurrence here is suggested by the time-dependent appearance of the bell-shaped isotherms (Fig. 7). Desensitization may potentially be achieved by receptor phosphorylation although, in preliminary experiments, a G-protein receptor kinase inhibitor (Ro318220, 1 µM) failed to block the descending phase of Gαi3 activation by 5-HT (Brzostowski and Kimmel, 2001; A. Newman-Tancredi, unpublished observations). Potential receptor conformational changes could also involve interaction with RGS proteins (for review, see Wieland and Chen, 1999), but regulatory mechanisms such as receptor internalization (Böhm et al., 1997) may be excluded, given that the present study was carried out using membrane preparations.

Conclusions

In conclusion, the present data demonstrate that i) h5-HT1A receptors couple efficiently to Gαi3 subunits; ii) GTP/ GDP exchange kinetics for Gαi3 subunits differ from those of other Gα subunits coupled to h5-HT1A receptors; iii) high concentrations of high-efficacy agonists suppress signaling to Gαi3. These differences in Gαi3 subunit activation constitute compelling evidence of agonist-dependent selection of different h5-HT1A receptor conformations. Further investigation is necessary to determine whether these phenomena are due to strength-of-signal mechanisms (such as differential efficiency of coupling to specific G-protein subtypes), agonist-directed trafficking, or receptor desensitization (Kenakin, 1995; Clarke and Bond, 1998). In addition, it would be of interest to investigate the consequences of these signaling patterns on downstream responses, such as adenylyl cyclase or MAP kinase, which may be modulated by multiple G-
protein subtypes (Raymond et al., 1993; Garnovskaya et al., 1997) and on the profiles of action of therapeutically-employed agents (Li et al., 1997). Finally, studies would be desirable to determine the extent of such G-protein activation patterns for other GPCRs, both in recombiant and native tissue membrane preparations.

References

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This work was supported by a grant from INSERM, PARIS, France. P. F. was supported in part by a fellowship from INRA, ile de France. The authors are grateful to A. Bruni for providing the human 5-HT1A receptor and to I. M., for stimulating discussion.

Agonist-Dependent Signaling at h5-HT1A Receptors


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