Differential Activation of Gq/11 and Gi3 Proteins at 5-Hydroxytryptamine2C Receptors Revealed by Antibody Capture Assays: Influence of Receptor Reserve and Relationship to Agonist-Directed Trafficking

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ABSTRACT

As determined by a guanosine 5’-O-(3-[35S]thio)triphosphate ([35S]GTPγS) binding assay, which does not distinguish G protein subtypes, 5-hydroxytryptamine (5-HT) and 2(S)-1-(6-chloro-5-fluoro-1H-indol-1-yl)-2-propanamine fumarate (Ro600175) behaved as full agonists at human 5-HT2C (h5-HT2C) receptors (VSV isoform) stably expressed in Chinese hamster ovary (CHO) cells, whereas 1–2,5-dimethoxy-4-iodophenyl-2-aminopropane (DOI), d-lysergic acid diethylamide (LSD), and lisuride exhibited partial agonist properties. After treatment with pertussis toxin to uncouple 5-HT2C receptors from Gi/Go but not Gq/11, DOI and LSD were as efficacious as 5-HT and Ro600175 in stimulating [35S]GTPγS binding, whereas lisuride still exhibited low efficacy (40%).

Correspondingly, in a scintillation proximity assay employing specific antibodies against Gq/11, 5-HT, Ro600175, DOI, and LSD behaved as high-efficacy agonists, whereas lisuride showed efficacy of 36%. In contrast, when employing a specific antibody recognizing Gi3, DOI and LSD were less efficacious (80 and 30%, respectively) than 5-HT and Ro600175, and lisuride was inactive. Agonist actions were specifically mediated by h5-HT2C receptors inasmuch as the selective 5-HT2C antagonist SB242,084 blocked [35S]GTPγS binding at both Gq/11 and Gi3. Agonist potency for stimulation of Gi3 was 6- to 8-fold less than for Gq/11, indicating that the latter was preferentially engaged by h5-HT2C receptors. Inactivation of h5-HT2C receptors with the alkyllating agent N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline did not modify the efficacy of 5-HT, Ro600175, and DOI at Gq/11, whereas their efficacies were substantially reduced at Gi3, indicating a greater receptor reserve for the former. Finally, the preferential activation of Gq/11 versus Gi3 by DOI, LSD, and lisuride was diminished in the presence of lower receptor number. In conclusion, h5-HT2C receptors couple to both Gq/11 and Gi3 in CHO cells, and efficacy for G protein subtype activation is both ligand- and receptor reserve-dependent.

5-HT2C receptors play a major role in the etiology and treatment of affective disorders, anxious states, schizophrenia, and Parkinson’s disease (Jenck et al., 1998; Fox and Brotchie, 1999; Meltzer, 1999). They are coupled to phospholipase C; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; h5-HT2C, human 5-HT2C; Ro600175, 2(S)-1-(6-chloro-5-fluoro-1H-indol-1-yl)-2-propanamine fumarate; LSD, d-lysergic acid diethylamide; DOI, 1-2,5-dimethoxy-4-iodophenyl-2-aminopropane.

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; CHO, Chinese hamster ovary; EEDQ, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; PLC, phospholipase C; PTX, pertussis toxin; [35S]GTPγS, guanosine-5’-O-(3-[35S]thio)triphosphate; SPA, scintillation proximity assay; PLA2, phospholipase A2; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; h5-HT2C, human 5-HT2C; Ro600175, 2(S)-1-(6-chloro-5-fluoro-1H-indol-1-yl)-2-propanamine fumarate; LSD, d-lysergic acid diethylamide; DOI, 1-2,5-dimethoxy-4-iodophenyl-2-aminopropane.
Materials and Methods

Membrane Preparation of CHO–h5-HT2C Cells. CHO cells stably expressing ~20 pmol mg−1 of edited h5-HT2C receptors (SVS isoform) were obtained from Euroscreen (Brussels, Belgium) and grown in adherent culture in 225-cm² flasks with UltraCHO medium (BioWhittaker Europe, Verviers, Belgium) containing sodium pyruvate (1 mM), dialyzed fetal calf serum (0.1%), and geneticin (400 µg/ml). Treatment of cells by pertussis toxin (Sigma-Aldrich, S. Quentin Fallavier, France) was performed overnight at 100 ng/ml. At confluence, cells were washed twice with buffer A containing 20 mM HEPES and 150 mM NaCl, pH 7.4. Cells were harvested from adherent culture and homogenized using a Kinematica Polytron homogenizer (20 s; Basel, Switzerland) in buffer A. The suspension was then centrifuged twice at 20,000g for 20 min at 4°C. The pellet was resuspended in buffer A, and an aliquot (~20 µg of protein per ml) was stored at −80°C until assay.

Competition Binding Assays. Binding affinity at h5-HT2C receptors was determined as described previously (Cussac et al., 2002) by competition binding with [3H]mesulergine (1 nM; Amersham Biosciences Inc., Saclay, France) in a buffer containing HEPES (20 mM), pH 7.7, EDTA (2 mM), and ascorbic acid (0.1% w/v). Incubations lasted 2 h at 22°C, and nonspecific binding was defined by 5-HT (10 µM). Isotherms were analyzed by nonlinear regression to yield IC50 values. Inhibition constants (K values) were derived from IC50 values according to Lazareno and Birdsall (1993).

Measurement of Agonist Efficacy and Antagonist Potency at h5-HT2C Receptors. Receptor-linked G protein activation by agonists at h5-HT2C receptors was determined by measuring the stimulation of [35S]GTPγS (1332 Ci/mmol; PerkinElmer Life Sciences, Paris, France) binding. CHO–h5-HT2C membranes (~20–30 µg per well) were preincubated 30 min with agonists and antagonists in a buffer B containing 20 mM HEPES, pH 7.4, 0.1 µM GDP, 50 mM MgCl2, and 150 mM NaCl, and reaction was started with 0.2 nM [35S]GTPγS in a final volume of 200 µl in 96-well plates for 60 min at room temperature. Experiments were terminated by rapid filtration through Unifilter-96 GF/B filters (PerkinElmer) using a Filtermate harvester (PerkinElmer Life Sciences, Boston, MA). Radioactivity retained on the filters was determined by liquid scintillation counting using a TopCount microplate scintillation counter (PerkinElmer Life Sciences). Agonist efficacy is expressed relative to 5-HT, which was tested at a maximal concentration in each experiment. Thus, basal binding (which includes both nonspecific radioactivity detection and endogenous guanine nucleotide turnover) is defined as 0%, whereas 5-HT-stimulated [35S]GTPγS binding is defined as 100%. All data are expressed as mean ± S.E.M. of at least three independent determinations.

Characterization of Antibodies Used in SPAs. To verify the specificity of the antibodies used in the SPA procedure, 25 ng of purified recombinant rat Gαo, Gαi1, Gαi2, Gαs, Gq, and Gα13 (Merck, Lidy, S.A., Fontenay sous Bois, France) were loaded on 10% polyacrylamide gel and transferred onto nitrocellulose. Immunoblotting of Gα subunits was performed using the polyclonal anti-Gαi1 (C19) from Santa Cruz Biotechnology (Santa Cruz, CA) (0.4 µg/ml) and the monoclonal antibody anti-Gαi3 from BIOMOL Research Laboratories (Plymouth Meeting, PA) (1 µg/ml), followed by enhanced chemiluminescence detection with horseradish peroxidase as secondary antibody (1/6000) (Amersham Biosciences Inc.).

Scintillation Proximity Assays. Specific activation of different subtypes of G proteins was determined using SPAs essentially as described by DeLapp et al. (1999). [35S]GTPγS binding was performed in the same conditions described above but in 96-well opti-plates (PerkinElmer Life Sciences). At the end of the incubation period, 20 µl of Nonidet P-40 (0.27% final concentration) was added to each well, and the plates were incubated with gentle agitation for 30 min. Antibodies specific for the G protein α-subunit of interest were then added to each well in a volume of 10 µl before 30 min of additional incubation period. The antibodies employed were the poly-
clonal anti-Gq/11 (1.74 μg/ml final dilution) and the monoclonal antibody anti-Gai1 (0.87 μg/ml final dilution). SPA beads coated with secondary anti-rabbit or anti-mouse antibodies from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK) were added in a volume of 50 μl at a dilution indicated by the manufacturer, and the plates were incubated for 3 h with gentle agitation. The plates were then centrifuged (10 min at 1300g), and radioactivity was detected on a TopCount microplate scintillation counter. Agonist efficacy is expressed relative to that of 5-HT, which was tested at a maximally effective concentration in each experiment (0.1 and 1 μM at Gq/11 and Gi1,3, respectively). All data are expressed as mean ± S.E.M. of at least three independent determinations.

5-HT2c Receptor Alkylation with EEDQ. CHO–h5-HT2c membranes were treated in buffer B with the alkylating agent, EEDQ, at a final concentration of 100 μM for 60 and 90 min at 30°C followed by SPA as described above. The percentage of maximal response as a function of the receptor occupancy (receptor reserve) was determined as described previously (Cussac et al., 2002). Briefly, plots were derived of 1/[A] versus 1/[A’]; where [A] and [A’] are equiactive concentrations for stimulation of [35S]GTPγS binding before and after receptor alkylation, respectively (90 min of EEDQ treatment for LSD effect at Gq/11 and 60 min of EEDQ treatment for 5-HT, Ro600175, and DOI effect at Gi3). K[A] values were determined by Furchgott analysis; K[A] = (slope-1)/y-intercept. Percentage receptor occupancy (O) was calculated by O = 100 × [L/(L + K[A])]; where L is the concentration of agonist. The curve is fitted by a rectangular hyperbola. All data are expressed as means ± S.E. of the mean of three independent determinations performed in triplicate. The level of h5-HT2c receptor expression after EEDQ treatment was determined by saturation experiments with [3H]mesulergine in parallel with SPA using the same membrane preparation. Protein concentration was determined colorimetrically using a bicinchoninic acid assay kit (Sigma-Aldrich).

Data Analysis. Isotherms were analyzed by nonlinear regression, using GraphPad Prism (GraphPad Software Inc., San Diego, CA) to yield EC50 and IC50 values. K[B] values of antagonists for inhibition of

![Fig. 1. Agonist stimulation of h5-HT2c receptor-mediated G protein activation.](image-url)
TABLE 1
Stimulation of [35S]GTPγS binding by agonist at h5-HT2C receptors

Agonist efficacies were determined by [35S]GTPγS binding at membrane preparation from CHO–h5-HT2C cells treated or not treated with pertussis toxin. Agonist efficacies are expressed relative to that of 5-HT (1 μM) determined in the absence of pertussis toxin and are means ± S.E.M. of at least three independent experiments.

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<th>With Pertussis Toxin</th>
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<tr>
<td></td>
<td>pEC50 (max)</td>
<td>E_{max} (max)</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>5-HT</td>
<td>8.08 ± 0.08 100.0 ± 5.7</td>
<td>8.64 ± 0.08 46.0 ± 4.2 (100)</td>
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<tr>
<td>Ro600175</td>
<td>7.80 ± 0.17 104.5 ± 5.3</td>
<td>8.52 ± 0.15 47.7 ± 2.5 (103)</td>
</tr>
<tr>
<td>DOI</td>
<td>7.72 ± 0.10 75.1 ± 6.4</td>
<td>8.05 ± 0.09 50.3 ± 3.6 (109)</td>
</tr>
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<td>LSD</td>
<td>8.06 ± 0.13 44.7 ± 4.9</td>
<td>8.00 ± 0.03 38.1 ± 2.3 (83)</td>
</tr>
<tr>
<td>Lisuride</td>
<td>7.47 ± 0.08 24.2 ± 2.8</td>
<td>7.76 ± 0.19 18.4 ± 3.2 (109)</td>
</tr>
</tbody>
</table>

5-HT-stimulated [35S]GTPγS binding were calculated according to Lazareno and Birdsell (1993): IC_{50} = IC_{50}/[1 + (Agonist/EC_{50})], where IC_{50} = inhibitory concentration_{50} of antagonist, agonist = concentration of 5-HT, and EC_{50} = effective concentration_{50} of 5-HT alone.

5-HT and N-ethoxy carbonyl-2-ethoxy-1,2-dihydroquinoline were purchased from Sigma-Aldrich. Ro600175 and SB242,084 [6-chloro-5-methyl-N-[6-(2-methylpyridin-3-yl)pyridin-3-yl]indoline-1-carboxamide] were synthesized by G. Lavielle (Institut de Recherches Servier). DOI and lisuride were purchased from Sigma/RBI (Natick, MA). LSD was supplied by Novartis (Basel, Switzerland).

**Results**

**[35S]GTPγS Binding at CHO–h5-HT2C Cell Membranes.** At CHO–h5-HT2C cell membranes, 5-HT elicited an increase in [35S]GTPγS binding typically by 1.4- to 1.6-fold with an EC_{50} of ~10 nM (Fig. 1; Table 1). The 5-HT_{2C} agonists, Ro600175, DOI, LSD, and lisuride, also stimulated [35S]GTPγS binding in a concentration-dependent manner, but only Ro600175 behaved as a full agonist compared with 5-HT (100%) (Fig. 1; Table 1). [35S]GTPγS binding induced by these agonists was abolished by the selective 5-HT_{2C} antagonist, SB242,084 (data not shown).

Pretreatment of CHO–h5-HT2C cells by PTX halved the induction of [35S]GTPγS binding by 5-HT and Ro600175. It also diminished, albeit to a lesser extent, the response to DOI. These observations demonstrate that h5-HT2C receptors coupled to both PTX-sensitive (Gi/Go proteins) and PTX-insensitive G proteins (Fig. 1; Table 1). In contrast, PTX treatment did not significantly affect [35S]GTPγS binding induced by LSD and lisuride (Fig. 1; Table 1). All agonists displayed similar efficacy for stimulation of 3H[GTPγS binding at PTX-insensitive G proteins, with the exception of lisuride, which exhibited partial agonist properties compared with 5-HT (Table 1). Moreover, the potencies of 5-HT, Ro600175, and DOI were increased by 2- to 5-fold by PTX cell treatment compared with untreated cells, whereas the potencies of LSD and lisuride were unaffected (Table 1).

** Specificity of Antibodies: Scintillation Proximity Assays Coupled to [35S]GTPγS Binding.** To address the issue of the precise nature of activated G protein in CHO-5-HT2C cells, we used two antibodies raised against Goa11 or Goa13 subunits. Fig. 2 shows that the monoclonal antibody anti-Goa13 did indeed recognize Goa13 but that it also cross-reacted with the Goa13 subunit. However, this antibody did not bind with other Go subunits of the Gi family (i.e., Goa2 and Goa) (Fig. 2). Previous studies of immunoreactive Ga

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**Fig. 2.** Immunodetection of Ga subunits. Purified Goa13, Goa13, Goa13, Goa, Goa, and Goa13 subunits were separated on a gel and submitted to immunodetection using polyclonal antibody anti-Goa11 (A) and the monoclonal antibody anti-Goa13 (B) as described under Materials and Methods.
GTP–branes from CHO. 

Materials and Methods

Representative experiments repeated on at least three occasions. Bars represent the mean of triplicate determinations from representative experiments repeated on at least three occasions.

**Fig. 3.** Agonist stimulation of h5-HT2C receptor-mediated activation of Gq/11 and Gi3 protein. Activation of G proteins was determined with anti-Gq/11 and anti-Gi3 protein antibodies captured, respectively, via secondary anti-rabbit or anti-mouse antibody-coated SPA beads as described under Materials and Methods. A and B, antagonist action of SB242,084 (1 μM) on 5-HT– (1 μM) and Ro600175-stimulated (1 μM) [35S]GTPγS binding at Gq/11 and Gi3 protein, respectively. C, [35S]GTPγS binding induced by 5-HT (10 nM) was performed on membranes from CHO–h5-HT2C cells treated or not treated with PTX. Results are expressed relative to basal values (100%). Actual basal values for Gq/11 in cpm were 1752 ± 62 in control and PTX-treated membranes, respectively, and actual basal values for Gi3 in cpm were 3206 ± 57 and 2516 ± 13 in control and PTX-treated membranes, respectively. Bars represent the mean of triplicate determinations from representative experiments repeated on at least three occasions.

**h5-HT2C Receptor Alkylation with EEDQ.** EEDQ treatment of membranes (100 μM, 30°C) time dependently decreased the density of h5-HT2C sites by 3.4- and 7.4-fold after 60 and 90 min of treatment, respectively, as determined by [3H]mesulergine saturation binding (Fig. 6). The KD value for [3H]mesulergine binding in control membranes (1.38 ± 0.13 nM) was unchanged by EEDQ treatment (1.37 ± 0.08 nM and 1.59 ± 0.15 nM for 60 and 90 min, respectively).

**Influence of h5-HT2C Receptor Alkylation with EEDQ On Gq/11 and Gi3 Activation.** The concentration-response curves of 5-HT–, Ro600175–, and DOI-mediated [35S]GTPγS binding to Gq/11 were progressively shifted to the right by treatment of CHO–h5-HT2C cells with EEDQ without a significant alteration in their relative efficacies, suggesting substantial receptor reserve (Fig. 7; Table 3). The KD value for LSD at Gq/11 determined by Furchgott analysis was 12.8 ± 3.6 nM. The derived occupancy/response yielded a linear plot with a half-maximal response to LSD at 48 ± 4.5% occupancy of h5-HT2C binding sites, demonstrating the absence of receptor reserve with this ligand for Gq/11 activation (Fig. 8). In the case of Gi3 activation, EEDQ treatment reduced both the potency of [35S]GTPγS binding by agonists as well as relative efficacies (Fig. 7; Table 3), suggesting a lower level of functional h5-HT2C receptors by EEDQ treatment (Fig. 7).

The selective 5-HT2C antagonist SB242,084 antagonized [35S]GTPγS binding induced by agonists in presence of PTX (Table 1). In contrast, Ro600175 and DOI exhibited lower efficacy than 5-HT at Gq/11 protein (P < 0.05, unpaired t test). LSD exhibited partial agonism (~30%) compared with 5-HT, lisuride being inactive at Gi3 proteins (Fig. 4; Table 2). Agonist potency for stimulation of Gi3 was about 6- to 8-fold less than for Gq/11 activation, except for LSD, which exhibited similar potency for activation of both G proteins (Table 2). The selective 5-HT2C antagonist SB242,084 antagonized 5-HT–stimulated [35S]GTPγS binding at Gq/11 and Gi3 proteins with similar potency (pKD values of 8.64 ± 0.04 and 8.69 ± 0.07, respectively; Fig. 5). LSD and lisuride diminished [35S]GTPγS binding at Gi3 and Gq/11 proteins, respectively, to a similar level as that obtained when the ligand was tested alone (Fig. 5). Antagonist potencies of lisuride at Gq/11 and Gi3 were 7.76 ± 0.10 and 7.25 ± 0.06, respectively, and LSD exhibited a pKD of 7.47 ± 0.05 at Gi3 (Fig. 5).
Fig. 4. Concentration-dependent agonist actions at h5-HT_{2C} receptor-mediated Gq/11 and Gi3 protein activation. Agonist concentration-response curves at Gq/11 (○) and Gi3 (●) from membrane preparation of CHO–h5-HT_{2C} cells. [35S]GTPγS binding is expressed as a percent of maximal stimulation with 5-HT (100%). Points shown are means of triplicate determinations from representative experiments repeated on at least three occasions. E_{max} and pEC_{50} data from these experiments are shown in Table 2.

TABLE 2
Stimulation of [35S]GTPγS binding by agonists at h5-HT_{2C} receptors coupled to Gq/11 and Gi3 and comparison with their respective affinities (pK_i values).

<table>
<thead>
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<th>Gq/11</th>
<th>Gi3</th>
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<tbody>
<tr>
<td></td>
<td>pEC_{50}</td>
<td>E_{max}</td>
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<tr>
<td>5-HT</td>
<td>8.67 ± 0.10</td>
<td>102.8 ± 2.2</td>
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<tr>
<td>Ro600175</td>
<td>8.44 ± 0.06</td>
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<tr>
<td>DOI</td>
<td>8.03 ± 0.03</td>
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</tr>
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<td>LSD</td>
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<td>Lisuride</td>
<td>7.44 ± 0.13</td>
<td>36.4 ± 5.5^*</td>
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N.C., not computable.

^pEC_{50} values were significantly lower (P < 0.05, unpaired t test) at Gi3.

^*E_{max} values differed significantly (P < 0.05, unpaired t test) versus 5-HT effect.
ysis yielded hyperbolic curves, with the half-maximal response for Gi3 activation being observed at h5-HT2C occupancies of 13.0 ± 3.7% for 5-HT, 21.6 ± 2.7% for Ro600175, and 33.7 ± 2.1% for DOI (Fig. 8). The receptor occupancy by DOI required to yield half-maximal Gi3 activation was significantly greater (P < 0.05, unpaired t test) than for 5-HT or Ro600175.

Discussion

The key findings of the present study are 1) the detection by [35S]GTPγS binding of 5-HT2C receptor-mediated activation of PTX-sensitive and -insensitive G proteins in CHO cell membranes, 2) the demonstration by an antibody capture/SPA detection strategy that 5-HT2C receptors coupled more efficiently to Gq/11 than to Gi3, and 3) as revealed by receptor alkylation, this difference reflects a high versus low level of receptor reserve for 5-HT2C receptors coupled to Gq/11 and Gi3, respectively.

[35S]GTPγS Binding at Whole-Cell Membrane Preparation: Influence of Pertussis Toxin. In cell membrane preparations of CHO cells expressing the VSV isoform of h5-HT2C receptors, 5-HT stimulated [35S]GTPγS binding. Although the selective 5-HT2C ligand Ro600175 also displayed high efficacy, DOI, LSD, and lisuride behaved as partial agonists. 5-HT-mediated [35S]GTPγS binding was sensitive to pertussis toxin, revealing the involvement of Gi/o proteins. This observation is consistent with reports that Gi/o proteins are involved in 5-HT2C receptor activation (see Introduction). Moreover, specific [35S]GTPγS binding to Gi has been observed in HEK 293 cells expressing 5-HT2C receptors (Alberts et al., 1999). However, although PTX reduced [35S]GTPγS binding, it did not abolish it, suggesting that agonists also stimulated PTX-insensitive G proteins. Agonist potencies and efficacies for stimulation of [35S]GTPγS binding in the presence of PTX (Table 1) were very similar to those observed for activation of Gq/11 as determined by SPA detection (see below; Table 2). However some [35S]GTPγS

![Fig. 5. Antagonism of h5-HT2C receptor-mediated Gq/11 and Gi3 protein activation. Antagonist concentration-response curves for SB242,084, LSD, and lisuride against 5-HT–stimulated [35S]GTPγS binding coupled to a scintillation proximity assay at Gq/11 (A) and Gi3 (B). Points shown are means of triplicate determinations from representative experiments repeated on at least three occasions. Antagonist potencies (pK_B values; see Results) were calculated from IC50 values for the inhibition of 5-HT–stimulated (10 nM for Gq/11 and 100 nM for Gi3) [35S]GTPγS binding.](image)

![Fig. 6. Time-dependent reduction of h5-HT2C receptor density by EEDQ. A, representative saturation binding isotherms of [3H]mesulergine to control CHO–h5-HT2C membranes compared with those pretreated with EEDQ (100 μM at 30°C for 60 and 90 min). B, Scatchard representation of data from A. Points shown are means of triplicate determinations from representative experiments repeated on three occasions. The mean B_max values were 18.5 ± 1.0 pmol mg^-1 without EEDQ and 5.4 ± 0.4 pmol mg^-1 and 2.5 ± 0.3 pmol mg^-1 in the presence of EEDQ for 60 and 90 min, respectively, without significant changes in K_D values.](image)
binding to G\textsubscript{q11} and G\textsubscript{i13} proteins, which can also couple to 5-HT\textsubscript{2C} receptors (Berg et al., 1999; Price et al., 2001), cannot be excluded. Thus, although direct labeling of G\textsubscript{q} by \([^{35}\text{S}]\text{GTP} \gamma \text{S} binding is difficult to detect, owing to low GTP-turnover rates (Smrcka et al., 1991), 5-HT\textsubscript{2C} receptors are able to activate \([^{35}\text{S}]\text{GTP} \gamma \text{S} binding to G\textsubscript{q11} proteins in CHO cells (present study) as well as in nonmammalian (insect Sf9) cells (Hartman and Northup, 1996).

Interestingly, because PTX did not affect the potency or efficacy of LSD and lisuride, we may surmise that these ligands mainly activated PTX-insensitive G proteins, probably G\textsubscript{q11}, in CHO–h5-HT\textsubscript{2C} cell membranes. Lisuride has previously been shown to exhibit antagonist properties at 5-HT\textsubscript{2C} receptors coupled to PLC in choroid plexus cells (Burris et al., 1991), whereas lisuride and LSD acted as weak partial agonists in NIH-3T3 cells (Egan et al., 1998). In the
present system, LSD behaved as a full agonist for stimulation of PTX-insensitive G proteins, in accordance with a study of inositol phosphate accumulation in HEK 293 cells expressing a high level of 5-HT2C receptors (Fitzgerald et al., 1999). However, the efficacy of lisuride represented about half that of LSD, in line with previous reports (Egan et al., 1998; Fitzgerald et al., 1999). Together with the finding that PTX treatment enhanced the potency of 5-HT, Ro600175, and DOI, these results suggest that h5-HT2C receptor activation preferentially engaged PTX-insensitive compared with PTX-sensitive G proteins.

5-HT2C Receptors Are Coupled to Gq/11 and Gi3 in CHO Cells: SPA Approach. Antibody capture/SPA detection using specific antibodies (see Results) allowed direct measurement of Gq/11 and Gi3 activation with a robust signal-to-noise ratio even after extensive receptor inactivation (see below). This SPA approach underpinned the above-discussed results concerning the in-

### TABLE 3

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<td>Gq/11</td>
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<td>5-HT</td>
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N.C., not computable.

*pEC50 and Emax values of agonists were significantly lower (P < 0.05, unpaired t test) in the presence of EEDQ (60 and 90 min) versus control.

*Emax and pEC50 values were significantly lower (P < 0.05, unpaired t test) in presence of EEDQ 90 min versus EEDQ 60 min.

5-HT2C. Receptors Are Coupled to Gq/11 and Gi3 in CHO Cells: SPA Approach. Antibody capture/SPA detection using specific antibodies (see Results) allowed direct measurement of Gq/11 and Gi3 activation with a robust signal-to-noise ratio even after extensive receptor inactivation by irreversible alkylation (see below). This SPA approach underpinned the above-discussed results concerning the in-
fluen
total $[^{35}S]$GTP$_\gamma$S binding in closely showing
that agonists at h5-HT$_{2C}$ (VSV) receptors preferentially engaged Gq/11. Activation of G proteins was specifically mediated by h5-HT$_{2C}$ receptors inasmuch as the selective 5-HT$_{2C}$ antagonist SB242,084 (Cussac et al., 2002) abolished 5-HT–stimulated $[^{35}S]$GTP$_\gamma$S binding at both Gq/11 and G$i_3$
subunits, excluding the involvement of endogenously expressed 5-HT$_{1H}$ receptors in CHO cells (Giles et al., 1996).

Although the hallucinogenic compound LSD (Glennon, 1996) did not exhibit different potencies in stimulating Gq/11 and G$i_3$, it behaved as a full agonist at the former and as a weak partial agonist at the latter. Furthermore, like lisuride, LSD antagonized 5-HT–induced stimulation of G$i_3$ (Fig. 5). Previous studies in HEK 293 cells have likewise shown that LSD is more efficacious at Gq (inositol generation) than at G$i/o$ ($[^{35}S]$GTP$_\gamma$S binding) (Alberts et al., 1999). However, although in the present study, 5-HT, RO600175, and DOI more potently activated Gq/11 than G$i_3$, the potency of 5-HT was similar at these G protein subtypes in the report of Alberts et al (1999). This distinction may be related to the use of a different isoform of h5-HT$_{2C}$ receptor, because it has been shown that editing of 5-HT$_{2C}$ receptors affects both PLC activation as well as G protein coupling (Backstrom et al., 1999; Berg et al., 2001; Price et al., 2001). The present differences in the actions of agonists at Gq/11 compared with G$i_3$ may influence trafficking of 5-HT$_{2C}$ receptor signaling at the effector level, as has been reported for stimulation of PLC and PLA$_2$ as well as cGMP production (Berg et al., 1998, 2001; Miller et al., 2000). Importantly, the application of antibody capture/SPA detection methodology to G proteins circumvents indirect effects, which may complicate interpretation of changes in signals downstream of G proteins. This includes effector crosstalk (PLC and PLA$_2$ sensitivity to $\beta\gamma$ subunits and Ca$^{2+}$, respectively), effector/receptor desensitization by protein kinase C (Cockcroft and Thomas, 1992), and direct actions of ligands at signals downstream to, or in parallel with, G proteins (Bockaert and Pin, 1999).

Receptor Reserve of h5-HT$_{2C}$ Receptor VSV Isoform Coupled to Gq/11 and G$i_3$. Recently, Brink et al (2000) reported that agonist-directed trafficking at human $\alpha_{2A}$-adrenoceptors is dependent on the level of receptor expression and, specifically, the presence of receptor reserve. Indeed, receptor number and receptor/G protein stoichiometry, as well as the specific identity of the G proteins activated, probably influence drug efficacies for activation of differing signaling cascades. For h5-HT$_{2C}$ receptors, no receptor reserve for PLC activation was demonstrated at elevated VSV and nonedited INI isoforms expressed in NIH-3T3 cells, despite the relatively high expression levels (~5 pmol/mg) (Burns et al., 1997). In contrast, the VNV isoform of h5-HT$_{2C}$ receptors expressed in HEK 293 exhibited receptor reserve for PLC activation, LSD behaving as a full agonist with increasing receptor number (Fitzgerald et al., 1999). Although these observations may reflect differential coupling between h5-HT$_{2C}$ receptor isoforms, variations in receptor/G protein stoichiometry are likely to be of major importance. Correspondingly, we investigated the influence of reducing receptor number (and therefore receptor/G protein stoichiometry) with the alkylating agent EEDQ.

In corroboration of our previous observations of the effect of EEDQ on h5-HT$_{2C}$ (VSV) receptor-mediated PLC activity (Cussac et al., 2002), we demonstrate herein that a high degree of receptor reserve exists for Gq/11 stimulation. Indeed, the potencies but not efficacies of 5-HT, Ro600175, and DOI for Gq/11 activation were reduced by EEDQ pretreatment, although the number of h5-HT$_{2C}$ receptors was diminished 7-fold (Fig. 6). In contrast, LSD exhibited partial agonist properties upon reduction of the density of functional h5-HT$_{2C}$ receptors. Accordingly, the occupancy/response plot of LSD for Gq/11 activation was linear, implying an absence of receptor reserve (Fig. 8), consistent with its partial agonist properties in other systems (Egan et al., 1998; Fitzgerald et al., 1999).

In contrast to Gq/11 activation, EEDQ markedly affected the efficacy of all agonists for h5-HT$_{2C}$ receptor-mediated G$i_3$ activation. Indeed, although hyperbolic occupancy/response plots were observed for 5-HT and Ro600175, the degree of receptor reserve was less than for activation of Gq/11. Moreover, in accordance with its partial agonist properties for Gi activation (Alberts et al., 1999), PLC activation (Fitzgerald et al., 1999), and Ca$^{2+}$ mobilization (Porter et al., 1999), DOI revealed a lower receptor reserve than 5-HT (Fig. 8). Thus, in the present system, potential agonist-directed trafficking by LSD and DOI must take into account the large receptor reserve for efficacious agonists, such as 5-HT and Ro600175. Thus, after 90-min EEDQ treatment of CHO–h5-HT$_{2C}$ membranes, pEC$_{50}$ values for agonist stimulation of Gq/11 were similar to pEC$_{50}$ values observed for G$i_3$ under control conditions. It is interesting to note that, under these conditions of “pEC$_{50}$ equivalence”, the (partial agonist) efficacy of LSD and DOI at Gq/11 approached that of G$i_3$. It may be concluded that the markedly higher efficacy of the partial agonists, DOI and LSD, at Gq/11 compared with G$i_3$ (observed in the absence of EEDQ) is associated with the more efficient coupling of h5-HT$_{2C}$ receptors to Gq/11 versus G$i_3$. Hence, caution should be exercised when differentiating potential agonist-directed trafficking from “strength-of-signal” mechanisms (for a review see Kenakin, 1995). In fact, agonist-directed trafficking implies a reordering of relative drug efficacies upon comparison of two G proteins (or, more generally, two effector pathways). In contrast, strength-of-signal mechanisms reflect receptor reserve, receptor/G protein stoichiometry, nature of expressed G proteins, etc. Thus, a strength-of-signal scheme, as well as agonist-directed trafficking, could account for the present observations. LSD and DOI (and lisuride) express their “agonist-directed trafficking” properties only under certain conditions of receptor reserve and/or receptor/G protein stoichiometry. A similar conclusion was reached by Brink et al. (2000) for $\alpha_{2A}$-adrenoceptor coupling to adenylyl cyclase via Gi and G$s$. Indeed, agonist-directed trafficking by 1-isoproterenol to Gs activation versus Gi was only observed under conditions where the full agonist, 5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalaminine, exhibited similar potency for activation of both G proteins.

These observations raise the question of whether receptor reserve exists for Gq/11 and/or G$i_3$ in physiological systems. In this context, it should be noted that coupling to G$i_3$ was observed herein even after extensive EEDQ treatment, reducing receptor expression to about 2 pmol/mg. In rat choroid plexus neurons, 5-HT$_{2C}$ expression levels (for a mixture of cell types) is also in the picomole(s) per milligram range (Yagaloff and Hartig, 1985), suggesting that the present data in CHO cells are relevant to central populations of 5-HT$_{2C}$.
receptors. Nevertheless, further investigation is needed to clarify the pertinence of the present data to other edited isoforms of h5-HT2C receptors. LSD, for example, did not stimulate PLC activity at the VGV isoform of 5-HT2C receptors (Backstrom et al., 1999; Berg et al., 2001). Applying the present antibody capture/SPA detection strategy to other h5-HT2C isoforms would enable this issue to be rapidly evaluated. Finally, it would be interesting to evaluate whether functional properties of LSD, DOI, and other agonists at h5-HT2C receptors may reflect their differential recruitment of specific G protein subtypes. Activation of 5-HT2A receptors, which couple to Gq/11 and PTX-sensitive G proteins (Kurra- s sch and Nichols, 2001), is associated with hallucinations, delusions, and many other effects (Glennon, 1996; Nelson et al., 1999). It would therefore be interesting to determine, in analogy with h5-HT2C receptors, whether LSD and DOI exhibit differential G protein activation at h5-HT2A receptors.

Conclusions

To summarize, the present study demonstrates that h5-HT2C receptors (VSV isoform) couple to both Gq/11 and Gq in CHO cells and that these G protein subtypes are recruited in an agonist- and receptor reserve-dependent manner. The differential influence of agonists on G protein coupling at h5-HT2C receptors may well be relevant to their functional profiles in vivo. It would be of interest to extend these data in characterizing the significance of receptor reserve, receptor/G protein stoichiometry, and G protein subtypes at other isoforms of h5-HT2C receptors as well as other classes of GPCR that couple to multiple intracellular signals.

References
