0026-895X/96/030474-07$3.00/0

MOLECULAR PHARMACOLOGY, 49:474–480 (1996).

Differences in Agonist-Independent and -Dependent 5-Hydroxytryptamine$_{2C}$ Receptor-Mediated Cell Division

RYAN S. WESTPHAL and ELAINE SANDERS-BUSH

Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Received August 30, 1995; Accepted November 7, 1995

SUMMARY

Previous studies have shown that agonist activation of the 5-hydroxytryptamine$_{2C}$ (5-HT$_{2C}$) receptor expressed in NIH-3T3 fibroblasts results in development of a transformed phenotype. In light of recent evidence from our laboratory demonstrating constitutive 5-HT$_{2C}$ receptor activity, we examined the contribution of this agonist-independent activity to basal cell division. 5-HT$_{2C}$ receptor ligands modulated [^{3}H]thymidine incorporation, DNA amounts, and cell number in serum-starved NIH-3T3 fibroblasts transfected with 5-HT$_{2C}$ receptor cDNA. Three classes of 5-HT$_{2C}$ receptor ligands were distinguished in transfected, but not nontransfected, fibroblasts. Basal [^{3}H]thymidine incorporation was increased by agonists and decreased by inverse agonists, whereas neutral antagonists had little or no effect alone. Neutral antagonists did, however, block the effects of both agonists and inverse agonists. The rank order of potencies of inverse agonists to decrease basal [^{3}H]thymidine incorporation was consistent with their rank order to decrease basal 5-HT$_{2C}$ receptor-mediated phosphoinositide hydrolysis. However, two antagonists previously classified as inverse agonists based on their ability to eliminate basal phosphoinositide hydrolysis did not elicit comparable reductions in basal [^{3}H]thymidine incorporation. For example, mesulergine had no effect on basal cell division, even though it eliminates the phosphoinositide hydrolysis response. Pertussis toxin, which inactivates G proteins in the G_i and G_o families, had no effect on basal [^{3}H]thymidine incorporation or basal phosphoinositide hydrolysis but partially inhibited these responses when elicited by an agonist. Thus, agonist occupation of the 5-HT$_{2C}$ receptor apparently activates different or additional G proteins compared with constitutive 5-HT$_{2C}$ receptor activation. In conclusion, our findings suggest that constitutively active 5-HT$_{2C}$ receptors stimulate cell division in transfected fibroblasts in the absence of an agonist. In addition, the 5-HT$_{2C}$ receptor may use multiple signaling pathways to mediate its effects.

The 5-HT$_{2C}$ receptor (formerly 5-HT$_{1C}$) is a member of the superfamily of G protein-coupled receptors (1) and is one of 15 receptor subtypes in the family of receptors for the neurotransmitter 5-HT (2). The 5-HT$_{2C}$ receptor is classified in the 5-HT$_2$ receptor subfamily based on amino acid sequence homology, signal transduction pathway, genomic organization, and pharmacological profile (2). 5-HT$_{2C}$ receptors were originally identified in membranes from choroid plexus by radioligand binding (3). More recently, *in situ* hybridization studies of 5-HT$_{2C}$ receptor mRNA have demonstrated that this receptor is widespread throughout the central nervous system (4, 5) and therefore may have an important role in mediating serotonergic functions. Furthermore, based on the clinical profiles of drugs that bind to the 5-HT$_{2C}$ receptor, this receptor is thought to have a role in mental disorders (6, 7), as well as hallucinogenesis (8).

Classically, G protein-coupled receptors are thought to require agonist occupation for activation of the signal transduction pathway. However, a growing body of evidence demonstrates that G protein-coupled receptors exhibit agonist-independent activation, termed constitutive receptor activity. Several wild-type (9–15) G protein-coupled receptors have been shown to exhibit constitutive activity. For example, the wild-type 5-HT$_{2C}$ receptor exhibits constitutive activation of PI hydrolysis when expressed in NIH-3T3 fibroblasts (15, 16) or Sf9 cells (17). Some 5-HT antagonists, termed inverse agonists, decrease agonist-independent 5-HT$_{2C}$ receptor activity. Other antagonists, termed neutral antagonists, do not reduce 5-HT$_{2C}$ receptor constitutive activity but block the effects of agonists and inverse agonists. Furthermore, agonists, inverse agonists, and neutral antagonists possess differential binding affinities for the G protein-coupled and -uncoupled forms of the 5-HT$_{2C}$ receptor (16). Agonists bind to the G protein-coupled form of the receptor with higher affinity than the uncoupled form, whereas inverse agonists bind the uncoupled form of the receptor with higher affinity. Neutral antagonists have equal affinity for

This work was supported by National Institutes of Health Grant MH34007 and Training Grant GM07628.

ABBREVIATIONS: PLC, phospholipase C; 5-HT, 5-hydroxytryptamine; BOL, (+)-2-bromolysergic acid diethylamide tartrate; (±)-DOI, (±)-1-(4-iodo-2,5-dimethoxyphenyl)-2-aminopropane; (−)-DOB, 1-(4-bromo-2,5-dimethoxyphenyl)-2-aminopropane; DMEM, Dulbecco's modified Eagle's medium; PI, phosphoinositide; HPLC, high performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

both receptor forms. Thus, both the receptor binding and receptor signaling properties of neutral antagonists are consistent with conventional receptor antagonists, whereas the properties of inverse agonists are opposite the properties of agonists.

Expression of either the 5-HT$_{2C}$ receptor (18) or the closely related 5-HT$_{2A}$ receptor (19) in NIH-3T3 fibroblasts elicits agonist-dependent mitogenesis and cell transformation. The mitogenic effects of some G protein-coupled receptors that activate PI hydrolysis are pertussis toxin sensitive (20–22) and may involve signaling pathways other than, or in addition to, PI hydrolysis (23, 24). We evaluated the role of agonist-independent 5-HT$_{2C}$ receptor activity in basal cell division of transfected NIH-3T3 fibroblasts as well as the sensitivity of 5-HT$_{2C}$ receptor-mediated mitogenesis to pertussis toxin. In the present study, we demonstrated that agonist-independent 5-HT$_{2C}$ receptor activation has functional consequences in cells. Furthermore, evidence is presented that agonist-mediated 5-HT$_{2C}$ receptor signaling activates additional, or different, G proteins compared with those activated by constitutive 5-HT$_{2C}$ receptor activity.

Experimental Procedures

Materials. NIH-3T3 fibroblasts were obtained from American Type Culture Collection (Rockville, MD). DMEM, penicillin, and streptomycin were purchased from GIBCO-BRL Life Technologies (Grand Island, NY). Calf serum was purchased from HyClone (Logan, UT). Cell culture dishes were purchased from Falcon/Becton Dickinson and Co. (Lincoln Park, NJ). Mianserin hydrochloride and (±)-DOI hydrochloride were purchased from Research Biochemicals (Natick, MA), atropine sulfate was purchased from Aldrich Chemical Company (Milwaukee, WI), and pertussis toxin was purchased from List Biologicals (Campbell, CA). BOL and (−)-DOB were obtained from the National Institute on Drug Abuse (Rockville, MD). Clozapine and methysergide maleate were gifts from Sandoz (East Hanover, NJ). [^{3}H]Thymidine (82 Ci/mmol), [^{32}P]NADH (30 Ci/mmol), and *myo*-[^{3}H]inositol (20–25 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA), and [^{3}H]mesulergine (82 Ci/mmol) was purchased from Amersham Corporation (Arlington Heights, IL). 5-HT creatinine sulfate, chlorpheniramine maleate, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture. NIH-3T3 fibroblasts cotransfected with an expression vector encoding the 5-HT$_{2C}$ receptor cDNA (1) and the selection vector pRSV/neo have been described previously (15). A subclone of this cell line, expressing 6.3 ± 1.6 pmol 5-HT$_{2C}$ receptor/mg protein, was used in the present study. NIH-3T3 fibroblasts were maintained in DMEM supplemented with 10% calf serum, penicillin (5 units/ml), and streptomycin (5 μg/ml) in a humidified incubator at 5% CO_2 at 37°.

[^{3}H]Thymidine incorporation. [^{3}H]Thymidine incorporation was assayed as described previously (25). Fibroblasts were plated in 15-mm wells with DMEM containing 10% calf serum. When cells were ~50% confluent, medium was changed to DMEM without serum for 24 hr. Cells were then incubated with fresh DMEM (without serum) containing 1 μCi [^{3}H]thymidine/ml. Appropriate drugs were added, and [^{3}H]thymidine incorporation was assayed 24 hr later. Medium was aspirated, cells were washed with cold phosphate-buffered saline, and fixed in methanol. After eight washes in tap water, nuclei and cytoskeletal remains were solubilized in 0.2 N NaOH/1% SDS for 2–3 hr at room temperature. The solubilized lysate was added to the scintillation cocktail, and radioactivity was quantified by liquid scintillation counting.

Quantification of cell number. Fibroblasts were plated in collagen-coated 35-mm wells with DMEM containing 10% calf serum. When cells reached 50% confluence, the medium was changed to DMEM without serum for 24 hr. Cells were then incubated with fresh DMEM (without serum) in the presence of appropriate drugs for 48 hr, with fresh drugs added each day. Subsequently, medium was aspirated, and cells were removed from the plates with incubation with Hanks' balanced salt solution containing 0.25% trypsin. Cell number was determined by counting aliquots of the cell suspension with a model Z_{BI} Coulter counter (Coulter Electronics).

Quantification of DNA. The amount of DNA in individual wells was determined by ethidium bromide assay (26) as modified (27). Cells were plated in 15-mm wells until they were ~50% confluent, serum starved for 24 hr, and then treated with appropriate drugs for 48 hr, with fresh drugs added each day. Forty-eight hours after initial drug treatment, medium was aspirated and cells were incubated for 30 min at 37° in buffer containing 0.25% trypsin, 10 mM Tris, 20 mM NaCl, and 5 mM Na_2EDTA, pH 7.4. Cells were sonicated, and heparin (2.5 units/ml) and ethidium bromide (2.5 μg/ml) were added and incubated for 30 min at room temperature. Fluorescence measurements were made on a Ratio Fluorometer-2 (Farrand Optical) with 350 nm used as the excitation wavelength and 585 nm used as the emission wavelength. Calf thymus DNA was used as the standard.

HPLC. HPLC analyses were performed as described previously (15) on serum-containing medium and conditioned medium. To determine the 5-HT concentration present in the conditioned medium, cells were serum starved for 24 hr, and medium was replaced and then collected 24 hr later for analysis of 5-HT content.

PI hydrolysis assay. The accumulation of inositol monophosphate was assayed based on the original method (28) with modifications (15). Briefly, NIH-3T3 fibroblasts transfected with 5-HT$_{2C}$ receptor cDNA were plated in 11-mm-diameter wells in DMEM containing 10% calf serum, penicillin, and streptomycin. Once confluent, cells were labeled with 1 μCi *myo*-[^{3}H]inositol/ml CMRL-1066 medium for 20–24 hr. Labeled cells were incubated for 10 min in the presence of 10 mM lithium chloride, 10 μM pargyline, and appropriate drugs. The reaction was terminated by aspiration of medium and the addition of methanol. [^{3}H]Inositol monophosphate was extracted with chloroform/methanol and isolated by anion exchange chromatography. Radioactivity was quantified by liquid scintillation counting.

Pertussis toxin ribosylation of membranes *in vitro*. Ribosylation assays were performed as described previously (29). NIH-3T3 fibroblasts transfected with 5-HT$_{2C}$ receptor cDNA were treated overnight with vehicle or pertussis toxin (100 ng/ml). Cells were then washed with phosphate-buffered saline and lysed in buffer consisting of 50 mM Tris·HCl, 5 mM $MgCl_2$, and 1 mM EDTA, pH 8.0, and centrifuged (15,000 × *g* for 15 min at 4°. Pellets were resuspended in the buffer and used in the ribosylation assays. Each reaction (100 μl volume) contained 200 μg of protein, 4 μCi of [^{32}P]NADH, 500 ng of pertussis toxin (preactivated at 37° for 30 min in the presence of 50 mM dithiothreitol), 1 mM ATP, 20 mM arginine, 20 mM thymidine, 100 mM NaCl, and 0.25% Lubrol. Samples were incubated at 30° for 40 min, reactions were terminated by addition of 1 ml HEPES (20 mM, pH 8.0), and then centrifuged 15,000 × *g* for 2 min. Pellets were resuspended in sample buffer (5% SDS, 25% glycerol, 150 mM Tris, pH 6.8, 1% β-mercaptoethanol) and subjected to SDS-polyacrylamide (12%) gel electrophoresis and autoradiography.

Results

5-HT$_{2C}$ receptor ligands modulate cell division. 5-HT$_{2C}$ receptor ligands were tested for their ability to alter [^{3}H]thymidine incorporation, DNA levels, and cell number (Fig. 1). Consistent with their effects on PI hydrolysis (15, 16), the agonist 5-HT increased, the inverse agonist mianserin decreased, and the neutral antagonist methysergide had minimal effect on [^{3}H]thymidine incorporation in NIH-

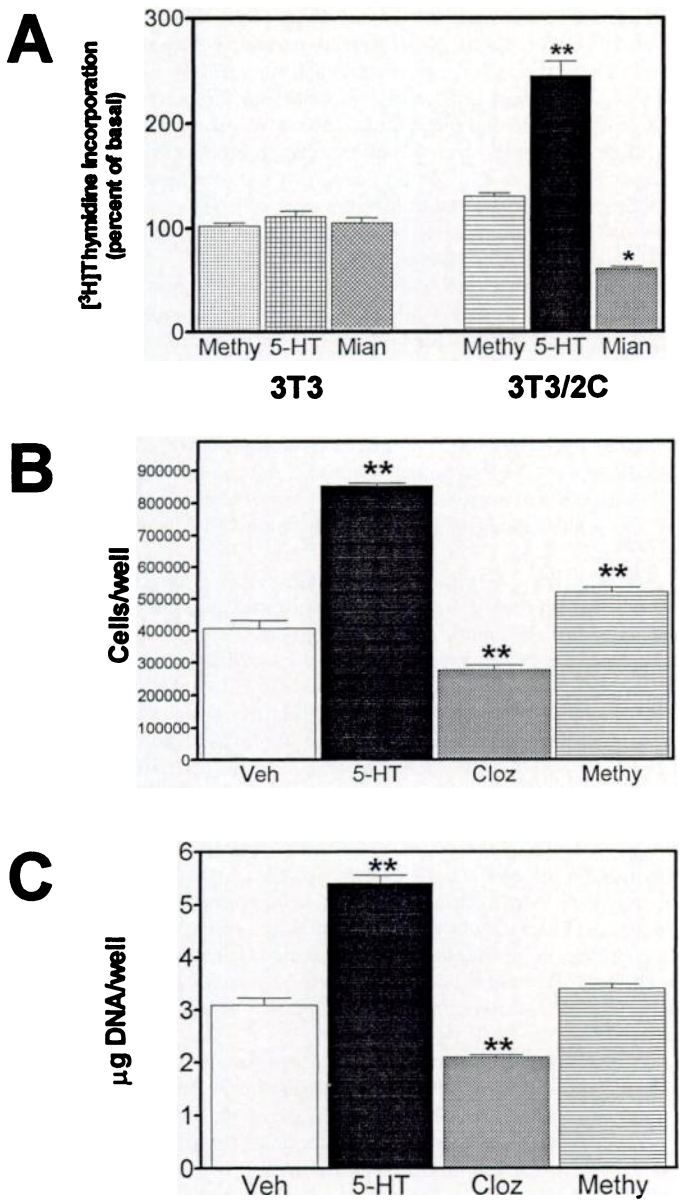

Fig. 1. Modulation of [^{3}H]thymidine incorporation, DNA levels, and cell number by 5-HT_{2C} receptor ligands. A, [^{3}H]Thymidine incorporation was measured in nontransfected NIH-3T3 fibroblasts and NIH-3T3 fibroblasts transfected with 5-HT_{2C} receptor cDNA as described in Experimental Procedures. Cells were treated with vehicle, 1 μM 5-HT, 1 μM mianserin (*mian*), or 1 μM methysergide (*methy*). Values in each experiment were normalized to the response in the presence of vehicle for each cell line. *Bars*, mean ± standard error of three to five experiments performed with quadruplicate determinations. Basal [^{3}H]thymidine incorporation ranged from 13,000 to 30,000 cpm/well. B, Cell numbers were determined in NIH-3T3 fibroblasts transfected with 5-HT_{2C} receptor cDNA as described in Experimental Procedures. Cells were treated with vehicle (*veh*), 1 μM 5-HT, 1 μM methysergide (*methy*), or 1 μM clozapine (*cloz*). *Bars*, mean ± standard error from six experiments. C, DNA levels were determined in NIH-3T3 fibroblasts transfected with 5-HT_{2C} receptor cDNA as described in Experimental Procedures. Cells were treated with vehicle (*veh*), 1 μM 5-HT, 1 μM methysergide (*methy*), or 1 μM clozapine (*cloz*). *Bars*, mean ± standard error from three experiments performed with triplicate determinations. Data were analyzed with one-way analysis of variance followed by Dunnett's *post hoc* test, in which each treatment is compared with vehicle (GraphPad InStat, La Jolla, CA). Statistically significant differences are indicated: *, $p < 0.05$; **, $p < 0.01$.

3T3 fibroblasts transfected with 5-HT_{2C} receptor cDNA. These drugs had no effect in nontransfected fibroblasts (Fig. 1A), suggesting that the effect on [^{3}H]thymidine incorporation was mediated by the 5-HT_{2C} receptor. Comparable changes in levels of DNA (Fig. 1C) and in cell number (Fig. 1B) were found in transfected cells treated with 5-HT, the inverse agonist clozapine, or methysergide. We therefore conclude that the effects of 5-HT_{2C} receptor ligands on [^{3}H]thymidine incorporation reflect changes in DNA synthesis and cell division.

5-HT_{2C} receptor antagonists decrease basal cell division. To determine whether constitutive 5-HT_{2C} receptor activity contributed to basal cell division, the ability of antagonists to decrease basal [^{3}H]thymidine incorporation was evaluated further in NIH-3T3 fibroblasts transfected with 5-HT_{2C} receptor cDNA. Clozapine, ketanserin, mianserin, and spiperone decreased agonist-independent [^{3}H]thymidine incorporation in a dose-dependent manner (Fig. 2), which is consistent with their classification as inverse agonists (15–17). Treatment for 24 hr with inverse agonists had no effect on total 5-HT_{2C} receptor density as measured by radioligand binding (data not shown), demonstrating that the inverse agonist-mediated decreases in basal [^{3}H]thymidine incorporation were independent of changes in total 5-HT_{2C} receptor density. The rank order of inverse agonist potencies to decrease [^{3}H]thymidine incorporation was consistent with the order of potency to decrease basal 5-HT_{2C} receptor-mediated PI hydrolysis (mianserin > clozapine > ketanserin > spiperone). The neutral antagonist BOL blocked the effects of the inverse agonists ketanserin (Fig. 3) and clozapine (data not shown), providing further evidence that the inverse agonist-mediated decreases in [^{3}H]thymidine incorporation were due to interaction with the 5-HT_{2C} receptor.

Mianserin seemed to function as a partial inverse agonist, eliciting a maximum decrease in [^{3}H]thymidine incorporation less than that elicited by the other inverse agonists (Fig. 2). Another putative inverse agonist, mesulergine, had no effect on basal [^{3}H]thymidine incorporation (Fig. 2) but

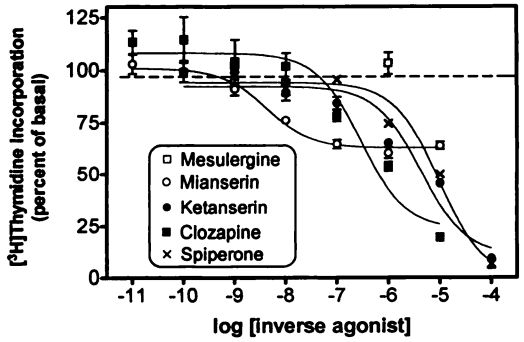

Fig. 2. Dose-dependent effects of 5-HT_{2C} receptor inverse agonists on basal [^{3}H]thymidine incorporation in NIH-3T3 fibroblasts transfected with 5-HT_{2C} receptor cDNA. Cells were treated with increasing concentrations of mianserin, clozapine, ketanserin, or spiperone or a single concentration of mesulergine and then assayed for [^{3}H]thymidine incorporation as described in Experimental Procedures. Values in each experiment were normalized to the response in the presence of vehicle. Values are the mean ± standard error of three experiments performed in triplicate. Basal [^{3}H]thymidine incorporation ranged from 23,000 to 80,000 cpm/well. Curves were plotted based on the equation for a sigmoid curve (GraphPad InPlot). Mean ± standard error EC_{50} values (three experiments) were mianserin, 2.1 ± 1 nM; clozapine, 82 ± 6 nM; ketanserin, 3.1 ± 0.8 μM; and spiperone, 7.9 ± 2 μM.

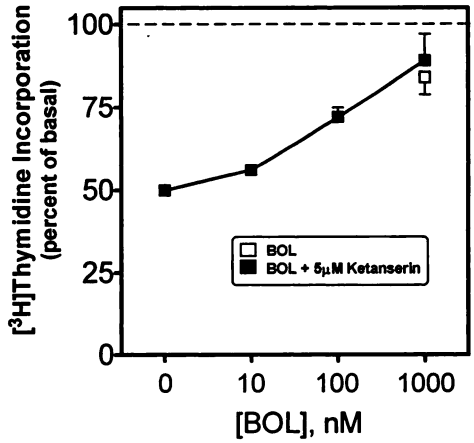

Fig. 3. Blockade of a 5-HT$_{2C}$ receptor inverse agonist by a neutral antagonist in NIH-3T3 fibroblasts transfected with 5-HT$_{2C}$ receptor cDNA. A, Cells were treated with vehicle plus 1000 nM BOL or with 5 μM ketanserin plus 0, 10, 100, or 1000 nM BOL and then assayed for [^{3}H]thymidine incorporation as described in Experimental Procedures. Values in each experiment were normalized to the basal response in the presence of vehicle alone. Basal [^{3}H]thymidine incorporation ranged from 30,000 to 44,000 cpm/well. Values represent the mean ± standard error of three experiments performed in triplicate.

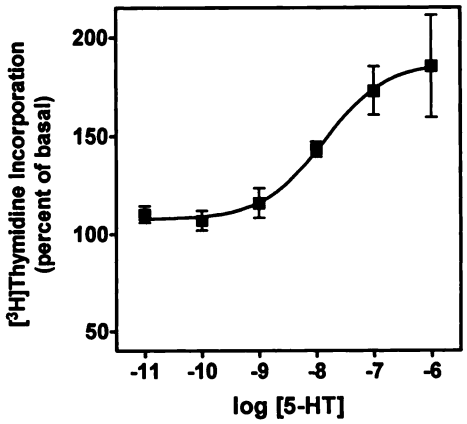

Fig. 4. Dose-dependent effects of 5-HT on [^{3}H]thymidine incorporation. NIH-3T3 fibroblasts transfected with 5-HT$_{2C}$ receptor cDNA were treated with increasing concentrations of 5-HT and assayed for [^{3}H]thymidine incorporation as described in Experimental Procedures. Values are the mean of three experiments performed in triplicate and are normalized to the maximum response after subtraction of the basal value. Basal [^{3}H]thymidine incorporation ranged from 2,600 to 71,000 cpm/well. EC_{50} values were determined by nonlinear regression analysis based on the equation for a sigmoid curve (GraphPad Prism, Intuitive Software for Science, La Jolla, CA). Mean ± standard error EC_{50} value (five experiments) for 5-HT was 11.6 ± 3 nM.

blocked the response to 5-HT (Table 1). These results with mianserin and mesulergine were unexpected, as these ligands are full inverse agonists with respect to PI hydrolysis (15).

Mechanism of 5-HT-mediated increases in [^{3}H]thymidine incorporation. 5-HT increased [^{3}H]thymidine incorporation with nanomolar potency (EC_{50} = 11.6 ± 3 nM) (Fig. 4). The 5-HT$_{2A/2C}$ receptor-specific agonists (±)-DOI and (−)-DOB (data not shown) potently increased [^{3}H]thymidine incorporation with EC_{50} values (three experiments) of 4 ± 2 and 7 ± 2 nM, respectively. The effects of 5-HT were blocked by the addition of 5-HT$_{2C}$ receptor inverse agonists and neutral antagonists but not by the muscarinic receptor antagonist atropine or the histamine receptor antagonist chlorpheniramine (Table 1). In addition, the 5-HT$_{1B}$ receptor antagonist pindolol had no effect on the potency (12.2 ± 7 nM; three experiments) or the maximum response (100 ± 4%; three experiments) of 5-HT-mediated [^{3}H]thymidine incorporation. Thus, pharmacological characterization of the agonist-mediated increase in [^{3}H]thymidine incorporation was consistent with the profile of the 5-HT$_{2C}$ receptor.

TABLE 1

Blockade of the 5-HT-mediated increase in [^{3}H]thymidine incorporation by 5-HT$_{2C}$ receptor inverse agonists and neutral antagonists

NIH/3T3 fibroblasts transfected with 5-HT$_{2C}$ receptor cDNA were treated with vehicle, 5-HT (10 nM), or 5-HT (10 nM) plus clozapine (1 μM), ketanserin (5 μM), BOL (1 μM), methysergide (1 μM), atropine (10 μM), or chlorpheniramine (10 μM) and assayed for [^{3}H]thymidine incorporation as described in Experimental Procedures. Values are mean ± standard error of three experiments performed with triplicate determinations normalized to the response in the presence of vehicle alone. Basal values were 17,000–63,000 cpm. Data were analyzed with one-way analysis of variance followed by Dunnett's *post hoc* test comparing each treatment with 5-HT alone (GraphPad InStat). *p* values are indicated (NS = not significant, $p > 0.05$).

Treatment	% Basal [^{3}H]thymidine incorporation	*p*
5-HT	162 ± 12	
5-HT plus:		
Methysergide	116 ± 1	0.01
BOL	83 ± 3	0.003
Ketanserin	77 ± 8	0.004
Clozapine	46 ± 2	0.002
Mesulergine	107 ± 6	0.01
Atropine	161 ± 11	NS
Chlorpheniramine	150 ± 16	NS

To gain insight into the classes of G proteins activated by the 5-HT$_{2C}$ receptor, the sensitivity to pertussis toxin was evaluated. 5-HT (Fig. 5A) and (−)-DOB (data not shown) -mediated increases in [^{3}H]thymidine incorporation and 5-HT-mediated increases in PI hydrolysis (Fig. 5B) were partially inhibited by pertussis toxin. In contrast, pertussis toxin had no effect on basal [^{3}H]thymidine incorporation or basal PI hydrolysis at concentrations as high as 500 ng pertussis toxin/ml (Fig. 5). The EC_{50} value for pertussis toxin attenuation of 5-HT-stimulated [^{3}H]thymidine incorporation (mean ± standard error; three experiments) was 0.4 ± 0.2 ng/ml (Fig. 5C), and the maximum inhibition was 20 ± 3%. To determine whether the partial effects of pertussis toxin were due to incomplete ADP-ribosylation, *in vitro* pertussis toxin-mediated ADP-ribosylation assays were performed after overnight treatment of cells with vehicle or pertussis toxin. These experiments demonstrated that overnight pertussis toxin treatment completely blocked subsequent *in vitro* pertussis toxin-mediated ADP-ribosylation (Fig. 5D).

Determination of the role of residual 5-HT in basal [^{3}H]thymidine incorporation. In previous studies, it has been established that agonist-independent 5-HT$_{2C}$ receptor activation is not due to 5-HT produced by NIH-3T3 fibroblasts or residual 5-HT from cell culture medium (15). The following results are consistent with that interpretation. Inverse agonists such as clozapine eliminated 90% of basal [^{3}H]thymidine incorporation in NIH-3T3 fibroblasts transfected with 5-HT$_{2C}$ receptor cDNA, demonstrating that 90% of the basal response was mediated by the 5-HT$_{2C}$ receptor. 5-HT elicited a maximal effect 100–150% above the basal response; therefore, contaminating 5-HT would have to be present at or near maximal concentrations to elicit the measured basal response. The concentration of 5-HT in serum-

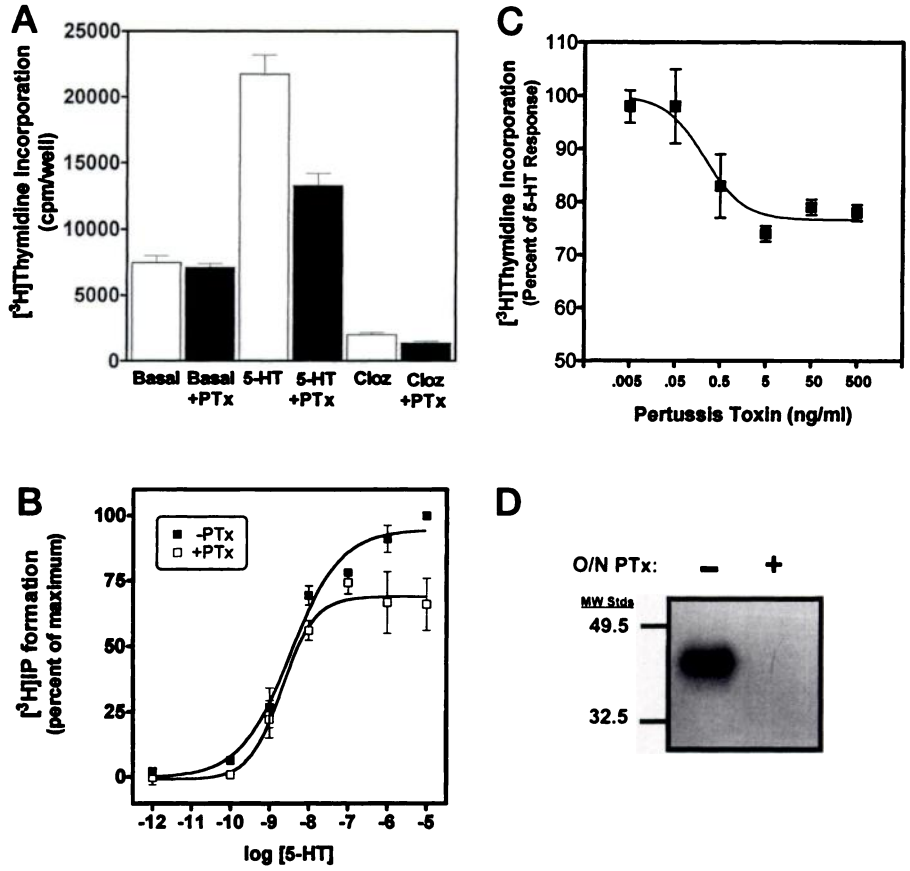

Fig. 5. Partial inhibition of agonist-stimulated 5-HT_{2C} receptor-mediated signaling by pertussis toxin. A, NIH-3T3 fibroblasts transfected with 5-HT_{2C} receptor cDNA were treated overnight with vehicle or 500 ng pertussis toxin/ml (*+PTx*) and then treated with vehicle (*Basal*), 1 μM 5-HT, or 1 μM clozapine (*Cloz*) and assayed for [^{3}H]thymidine incorporation as described in Experimental Procedures. Values are mean ± standard error from a single experiment performed in triplicate and are representative of three separate experiments. B, *myo*-[^{3}H]Inositol-labeled NIH-3T3 fibroblasts transfected with 5-HT_{2C} receptor cDNA were treated overnight with vehicle (■) or 5 ng/ml pertussis toxin (□) and then treated with increasing concentrations of 5-HT and assayed for PI hydrolysis as described in Experimental Procedures. Values are mean ± standard error and from three separate experiments performed in duplicate, normalized to the maximum response of 5-HT in the absence of pertussis toxin. EC_{50} values (mean ± standard error; three experiments) were without pertussis toxin, 3.7 ± 1.3 nM; and with pertussis toxin, 2.5 ± 1.3 nM. E_{max} value (mean ± standard error; three experiments) after pertussis toxin treatment was 74 ± 6% of the E_{max} after treatment with vehicle ($p < 0.05$). The basal level of PI hydrolysis after pertussis toxin treatment was 89 ± 7% of basal level after vehicle treatment ($p > 0.05$). The basal level of PI hydrolysis after treatment with 500 ng pertussis toxin/ml was 98 ± 9% of basal level after treatment with vehicle ($p > 0.05$), and the E_{max} of 5-HT-stimulated PI hydrolysis after treatment with 500 ng pertussis toxin/ml was 73 ± 5% of the E_{max} after treatment with vehicle ($p < 0.05$). Data were analyzed with the use of the paired Student's *t* test (GraphPad InStat). C, NIH-3T3 fibroblasts transfected with 5-HT_{2C} receptor cDNA were treated overnight with vehicle or increasing concentrations of pertussis toxin and then treated with 1 μM 5-HT and assayed for [^{3}H]thymidine incorporation as described in Experimental Procedures. Values are mean ± standard error and from a single experiment performed in triplicate and are representative of three separate experiments. D, NIH-3T3 fibroblasts transfected with 5-HT_{2C} receptor cDNA were treated overnight with vehicle or pertussis toxin (100 ng/ml) and then subjected to *in vitro* pertussis toxin-ribosylation assays as described in Experimental Procedures. *Lane 1*, overnight treatment with vehicle (*O/N PTx* −). *Lane 2*, overnight treatment with pertussis toxin (*O/N PTx* +). Presented is a representative autoradiograph (two experiments). MW Stds = molecular mass standards × 10^3.

containing medium was 19 nM. However, extensive washing that mimicked the [^{3}H]thymidine incorporation assay reduced 5-HT to an undetectable level, based on HPLC assays that could detect a concentration as low as 0.25 nM. Because the EC_{50} of 5-HT was 14 nM, 0.25 nM 5-HT could not cause the observed basal response. A second strategy to estimate the concentration of 5-HT present in the assay medium was to quantify the dilution of added [^{3}H]-5-HT (668,063 ± 13,754 cpm/500 μl serum-containing medium). To mimic the [^{3}H]thymidine incorporation assay, cells were incubated in [^{3}H]-5-HT-containing medium for 24 hr, in fresh DMEM (without serum or [^{3}H]-5-HT) for 24 hr, and then in fresh medium again. The [^{3}H]-5-HT in the final medium was quantified by liquid scintillation counting. Extremely low levels of [^{3}H]-5-HT were present in this medium (145 ± 8 cpm/500 μl). The dilution of [^{3}H]-5-HT was calculated to be 4639 ± 318-fold. Assuming that the dilution of unlabeled-5-HT in serum mimicked the dilution of [^{3}H]-5-HT, the final concentration in the assay medium would be <5 pM. These data are consistent with the HPLC analyses of 5-HT content in conditioned medium and confirm that residual 5-HT did not account for basal [^{3}H]thymidine incorporation.

Discussion

Expression of the 5-HT_{2C} receptor in fibroblasts results in receptor-mediated PI hydrolysis that occurs in the absence of agonist, a property termed constitutive receptor activity (15). Constitutive 5-HT_{2C} receptor activity is decreased by many 5-HT_{2C} receptor antagonists with negative intrinsic activity

(15–17). These drugs are referred to as inverse agonists or negative antagonists. Another group of antagonists, termed neutral antagonists, block the effects of agonists and inverse agonists but have little or no effect on constitutive receptor activity (15). The property of constitutive activity is not unique to the 5-HT$_{2C}$ receptor but rather has also been demonstrated for a number of other G protein-coupled receptors, including the α_2-adrenergic (12), β_2-adrenergic (11), D_{1B}-dopaminergic (13), B_2 bradykinin (14), and δ opioid receptors (9, 10).

In the current study, we demonstrated that expression of 5-HT$_{2C}$ receptors in fibroblasts resulted in high levels of [^{3}H]thymidine incorporation in the absence of agonist. This conclusion is based on the following findings: some 5-HT$_{2C}$ receptor antagonists decreased basal activity, whereas other antagonists did not alter basal activity, even though they blocked the effects of agonists. Furthermore, HPLC and dilution analyses showed that the concentration of 5-HT in conditioned medium could not account for the basal level of [^{3}H]thymidine incorporation. The finding that inverse agonists did not alter [^{3}H]thymidine incorporation in nontransfected cells, combined with data showing that a neutral antagonist blocked the effects of inverse agonists, strongly suggests that inverse agonists decreased [^{3}H]thymidine incorporation in transfected cells via interaction with the 5-HT$_{2C}$ receptor. In addition, an inverse agonist reduced DNA levels and cell number, suggesting that agonist-independent 5-HT$_{2C}$ receptor activity contributes to fibroblast cell division and transformation.

We also demonstrated that agonist activation of the 5-HT$_{2C}$ receptor increased basal [^{3}H]thymidine incorporation in NIH-3T3 fibroblasts, which is consistent with the observation that 5-HT$_{2C}$ receptor activation results in fibroblast transformation (18). Pharmacological characterization of agonist-mediated increases in cell division was in agreement with the pharmacological profile of the 5-HT$_{2C}$ receptor. In addition, 5-HT$_{2C}$ receptor agonists had no effect in nontransfected cells, which is consistent with the interpretation that the mitogenic effects were mediated through the 5-HT$_{2C}$ receptor. The 5-HT$_{1B}$ receptor has been shown to function as a mitogen in synergy with growth factors in some fibroblast cell lines (21); however, the following data suggest that the 5-HT$_{1B}$ receptor does not have a role in the current experiments. First, neither the mitogenic effects of 5-HT nor basal [^{3}H]thymidine incorporation was reduced by the 5-HT$_{1B}$ receptor antagonist pindolol. Second, the present experiments were performed in the absence of other growth factors, a requirement for a 5-HT$_{1B}$ receptor-mediated mitogenesis (21). Last, the 5-HT$_2$ receptor-selective agonists (−)-DOB and (±)-DOI (30–32) potently increased [^{3}H]thymidine incorporation. Therefore, the actions of 5-HT that we describe seem to be independent of 5-HT$_{1B}$ receptor activation.

Pertussis toxin partially inhibited the 5-HT-mediated increases in [^{3}H]thymidine incorporation and PI hydrolysis. It is not known whether the 5-HT$_{2C}$ receptor couples to pertussis toxin-sensitive or -insensitive G proteins *in vivo*; however, the partial inhibitory effect of pertussis toxin in NIH-3T3 fibroblasts suggests that the 5-HT$_{2C}$ receptor has the ability to couple to both classes of G proteins. This conclusion is consistent with studies of the expression of the cloned 5-HT$_{2C}$ receptor in *Xenopus* oocytes, which also exhibited partial sensitivity to pertussis toxin (33). In the present study, only the agonist responses were attenuated by pertussis toxin treatment; basal receptor activity was not altered. Thus, it seems that agonist activation of the 5-HT$_{2C}$ receptor uses different or additional G proteins that are not involved in constitutive receptor activity. This is the first evidence that the signaling pathways involved in agonist activation of G protein-coupled receptors may differ from the pathways activated by the constitutively active form of the receptor.

Interestingly, the inverse agonists mianserin and mesulergine had partial or no effect on basal [^{3}H]thymidine incorporation. This is in contrast with their ability to eliminate basal 5-HT$_{2C}$ receptor-mediated PI hydrolysis (15). The [^{3}H]thymidine incorporation assay required a 24-hr incubation with drugs compared with a 45-min incubation in the PI hydrolysis assay. It was possible, therefore, that degradation of drug could be responsible for the attenuated effects of mianserin and mesulergine on [^{3}H]thymidine incorporation. However, mesulergine blocked completely the 5-HT-mediated increase in [^{3}H]thymidine incorporation, suggesting that degradation of mesulergine during the assay was not a factor. The finding that mianserin and mesulergine exhibit partial or no effect in [^{3}H]thymidine incorporation assays, despite their full inverse-agonist property in PI hydrolysis assays, suggests that the 5-HT$_{2C}$ receptor may activate signaling pathways other than or in addition to PI hydrolysis to elicit cell division. This conclusion is consistent with the evidence that the 5-HT$_{2C}$ receptor may activate different signaling pathways to elicit basal and agonist-mediated responses.

The present demonstration of mitogenic effects associated with constitutive activity of the 5-HT$_{2C}$ receptor suggests that ectopic expression of the 5-HT$_{2C}$ receptor has the capacity to elicit cellular transformation and tumor formation, even in the absence of 5-HT. In addition, spontaneous 5-HT$_{2C}$ receptor activity may be important in brain development, stimulating cell growth before innervation by serotonergic fibers. The findings that agonist-mediated, but not constitutive, receptor activity is partially sensitive to pertussis toxin and that some inverse agonists eliminate PI hydrolysis, but not [^{3}H]thymidine incorporation, suggest that 5-HT$_{2C}$ receptor signaling may be more complex than previously recognized (34). Indeed, recent work has shown that 5-HT$_{2C}$ receptor activity increases the level of intracellular cGMP (35) and also blocks 5-HT$_{1B}$ receptor-mediated decreases in cAMP (36), although it is not clear whether these signals are independent of or downstream from PI hydrolysis. In future studies of 5-HT$_{2C}$ receptor signal transduction, the role of the various pathways should be evaluated in the function of constitutively activated as well as agonist-activated 5-HT$_{2C}$ receptors.

Acknowledgments

The authors acknowledge Dr. David Julius (University of California, San Francisco) for providing the 5-HT$_{2C}$ receptor cDNA, Dr. Dennis Schmidt (Department of Psychiatry, Vanderbilt University, Nashville, TN) for analyses of 5-HT, Dr. Michael S. Grotewiel (Department of Pharmacology, Vanderbilt University, Nashville, TN) for helpful discussions, and Ann Westphal (Department of Pharmacology, Vanderbilt University, Nashville, TN) for expert technical assistance.

References

1. Julius, D., A. B. MacDermott, R. Axel, and T. M. Jessell. Molecular characterization of a functional cDNA encoding the serotonin-1c receptor. *Science (Washington D. C.)* **241**:558–564 (1988).
2. Hoyer, D., D. E. Clarke, J. R. Fozard, P. R. Hartig, G. R. Martin, E. J. Mylecharane, P. R. Saxena, and P. P. A. Humphrey. VII. International Union of Pharmacology classification of receptors for 5-hydroxytryptamine (serotonin). *Pharmacol. Rev.* **46**:157–203 (1994).
3. Pazos, A., D. Hoyer, and J. M. Palacios. The binding of serotonergic ligands to the porcine choroid plexus: characterization of a new type of serotonin recognition site. *Eur. J. Pharmacol.* **106**:539–546 (1984).
4. Molineaux, S. M., T. M. Jessell, R. Axel, and D. Julius. 5-HT1c receptor is a prominent serotonin receptor subtype in the central nervous system. *Proc. Natl. Acad. Sci. USA* **86**:6793–6797 (1989).
5. Hoffman, B. J., and E. Mezey. Distribution of serotonin 5-HT1C receptor mRNA in adult rat brain. *FEBS Lett.* **247**:453–462 (1989).
6. Kennett, G. A., P. Whitton, K. Shah, and G. Curzon. Anxiogenic-like effects of mCPP and TFMPP in animal models are opposed by 5-HT1C receptor antagonists. *Eur. J. Pharmacol.* **164**:445–454 (1989).
7. Canton, H., L. Verriele, and F. C. Colpaert. Binding of typical and atypical antipsychotics to 5-HT1C and 5-HT2 sites: clozapine potently interacts with 5-HT1C sites. *Eur. J. Pharmacol.* **191**:93–96 (1990).
8. Sanders-Bush, E., and M. Breeding. Choroid plexus epithelial cells in primary culture: a model of 5HT1C receptor activation by hallucinogenic drugs. *Psychopharmacology* **105**:340–346 (1991).
9. Costa, T., and A. Herz. Antagonists with negative intrinsic activity at *delta* opioid receptors coupled to GTP-binding proteins. *Proc. Natl. Acad. Sci. USA* **86**:7321–7325 (1989).
10. Costa, T., J. Lang, C. Gless, and A. Herz. Spontaneous association between opioid receptors and GTP-binding regulatory proteins in native membranes: specific regulation by antagonists and sodium ions. *Mol. Pharmacol.* **37**:383–394 (1990).
11. Chidiac, P., T. E. Hebert, M. Valiquette, M. Dennis, and M. Bouvier. Inverse agonist activity of β-adrenergic antagonists. *Mol. Pharmacol.* **45**: 490–499 (1994).
12. Tian, W. N., E. Duzic, S. M. Lanier, and R. C. Deth. Determinants of α_2-adrenergic receptor activation of G proteins: evidence for a precoupled receptor/G protein state. *Mol. Pharmacol.* **45**:524–531 (1994).
13. Tiberi, M., and M. G. Caron. High agonist-independent activity is a distinguishing feature of the dopamine D1B receptor subtype. *J. Biol. Chem.* **269**:27925–27931 (1994).
14. Leeb-Lundberg, F. L. M., S. A. Mathis, and M. C. S. Herzig. Antagonists of bradykinin that stabilize a G-protein-uncoupled state of the B2 receptor act as inverse agonists in rat myometrial cells. *J. Biol. Chem.* **269**:25970–25973 (1994).
15. Barker, E. L., R. S. Westphal, D. Schmidt, and E. Sanders-Bush. Constitutively active 5-hydroxytryptamine 2C (5HT2C) receptors reveal novel inverse agonist activity of receptor ligands. *J. Biol. Chem.* **269**:11687–11690 (1994).
16. Westphal, R. S., and E. Sanders-Bush. Reciprocal binding properties of 5-hydroxytryptamine type 2C receptor agonists and inverse agonists. *Mol. Pharmacol.* **46**:937–942 (1994).
17. Labrecque, J., A. Fargin, M. Bouvier, P. Chidiac, and M. Dennis. Serotonergic antagonists differentially inhibit spontaneous activity and decrease ligand binding capacity of the rat 5-hydroxytryptamine type 2C receptor in Sf9 cells. *Mol. Pharmacol.* **48**:150–159 (1995).
18. Julius, D., T. J. Livelli, T. M. Jessell and R. Axel. Ectopic expression of the serotonin 1c receptor and the triggering of malignant transformation. *Science (Washington D. C.)* **244**:1057–1062 (1989).
19. Julius, D., K. N. Huang, T. J. Livelli, R. Axel, and T. M. Jessell. The 5HT2 receptor defines a family of structurally distinct but functionally conserved serotonin receptors. *Proc. Natl. Acad. Sci. USA* **87**:928–932 (1990).
20. Zachary, I., J. Millar, E. Nanberg, T. Higgins, and E. Rozengurt. Inhibition of bombesin-induced mitogenesis by pertussis toxin: dissociation from phospholipase C pathway. *Biochem. Biophys. Res. Commun.* **146**:456–463 (1987).
21. Chambard, J. C., S. Paris, G. L'Allemain, and J. Pouyssegur. Two growth factor signalling pathways in fibroblasts distinguished by pertussis toxin. *Nature (Lond.)* **326**:800–803 (1987).
22. Letterio, J. J., S. R. Coughlin, and L. T. Williams. Pertussis toxin-sensitive pathway in the stimulation of c-myc expression and DNA synthesis by bombesin. *Science (Washington D. C.)* **234**:1117–1119 (1986).
23. Seuwen, K., C. Kahan, T. Hartmann, and J. Pouyssegur. Strong and persistent activation of inositol lipid breakdown induces early mitogenic events but not Go to S phase progression in hamster fibroblasts. *J. Biol. Chem.* **265**:22292–22299 (1990).
24. Crespo, P., N. Xu, J. L. Daniotti, J. Troppmair, U. R. Rapp, and J. S. Gutkind. Signaling through transforming G protein-coupled receptors in NIH 3T3 cells involves c-Raf activation. *J. Biol. Chem.* **269**:21103–21109 (1994).
25. Esterle, T. M., and E. Sanders-Bush. Serotonin agonists increase transferrin levels via activation of 5-HT1C receptors in choroid plexus epithelium. *J. Neurosci.* **12**:4775–4782 (1992).
26. Karsten, U., and A. Wollenberger. Improvements in the ethidium bromide method for direct fluorometric estimation of DNA and RNA in cell and tissue homogenates. *Anal. Biochem.* **77**:464–470 (1977).
27. Skinner, M. K., P. M. Fetterolf, and C. T. Anthony. Purification of a paracrine factor, P-Mod-S, produced by testicular peritubular cells that modulates Sertoli cell function. *J. Biol. Chem.* **263**:2884–2890 (1988).
28. Berridge, M. J., C. P. Downes, and M. R. Hanley. Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. *Biochem. J.* **206**:587–595 (1982).
29. Grotewiel, M. S., H. Chu, and E. Sanders-Bush. m-Chlorophenylpiperazine and m-trifluoromethylphenylpiperazine are partial agonists at cloned 5-HT2A receptors expressed in fibroblasts. *J. Pharmacol. Exp. Ther.* **271**: 1122–1126 (1994).
30. Shannon, M., G. Battaglia, R. A. Glennon, and M. Titeler. 5-HT1 and 5-HT2 binding properties of derivatives of the hallucinogen 1-(2,5-dimethoxyphenyl)-2-aminopropane (2,5-DMA). *Eur. J. Pharmacol.* **102**: 23–29 (1984).
31. Titeler, M., R. A. Lyon, K. H. Davis, and R. A. Glennon. Selectivity of serotonergic drugs for multiple brain serotonin receptors. *Biochem. Pharmacol.* **36**:3265–3271 (1987).
32. Titeler, M., R. A. Lyon, and R. A. Glennon. Radioligand binding evidence implicates the brain 5-HT2 receptor as a site of action for LSD and phenylisopropylamine hallucinogens. *Psychopharmacology* **94**:213–216 (1988).
33. Quick, M. W., M. I. Simon, N. Davidson, H. A. Lester, and A. M. Aragay. Differential coupling of G protein *alpha* subunits to seven-helix receptors expressed in *Xenopus* oocytes. *J. Biol. Chem.* **269**:30164–30172 (1994).
34. Sanders-Bush, E., and H. Canton. Serotonin receptors signal transduction pathways, in *Psychopharmacology: The Fourth Generation of Progress* (F. E. Bloom and D. J. Kupfer, eds.). Raven Press, New York, 431–441 (1995).
35. Kaufman, M. J., P. R. Hartig, and B. J. Hoffman. Serotonin 5-HT2C receptor stimulates cyclic GMP formation in choroid plexus. *J. Neurochem.* **64**:199–205 (1995).
36. Berg, K. A., W. P. Clarke, C. Sailstad, A. Saltzman, and S. Maayani. Signal transduction differences between 5-hydroxytryptamine type 2A and type 2C receptor systems. *Mol. Pharmacol.* **46**:477–484 (1994).

Send reprint requests to: Dr. Elaine Sanders-Bush, 461 MRB II, Department of Pharmacology, Vanderbilt University, Nashville, TN 37232. E-mail: bushes@ctrvax.vanderbilt.edu