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Reciprocal Binding Properties of 5-Hydroxytryptamine Type 2C Receptor Agonists and Inverse Agonists

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SUMMARY

Expression of the 5-hydroxytryptamine type 2C (5-HT_{2C}) receptor in NIH/3T3 fibroblasts results in agonist-independent 5-HT_{2C} receptor activation. Some 5-HT_{2C} receptor antagonists decrease this activation and are termed inverse agonists. The present study uses this system to evaluate functional and receptor binding properties of other 5-HT_{2C} receptor antagonists. A number of inverse agonists, including clozapine, and a neutral antagonist (methysergide) were identified in a functional assay. Guanine nucleotides increased the affinity of a radiolabeled inverse agonist ([^{3}H]mesulergine), suggesting that inverse agonists bind the G protein-uncoupled form of the 5-HT_{2C} receptor with high affinity. Competition binding was performed using conditions that separately labeled the G protein-coupled and -uncoupled forms of the receptor. These studies demonstrated that inverse agonists bound the uncoupled form of the 5-HT_{2C} receptor with higher affinity, compared with the G protein-coupled form. Agonists, on the other hand, had higher affinity for the coupled form, whereas neutral antagonists had equal affinity for both forms of the receptor. Thus, 5-HT_{2C} receptor neutral antagonists exhibited functional and receptor binding properties consistent with those of classical receptor antagonists. However, 5-HT_{2C} receptor inverse agonists displayed functional and receptor binding properties that were opposite those of agonists.

Classical models of G protein-coupled receptors require agonist occupation of the receptor to activate signal transduction pathways. However, studies using reconstituted receptors (1) and membrane preparations (2–4) demonstrate that receptor activation also occurs in the absence of agonist. In addition, recent studies in intact cells show that some mutant (5–7) as well as wild-type (8, 9) receptors exhibit constitutive activity, defined as agonist-independent receptor activation. In some of these systems, constitutive receptor activity is reduced by antagonists (2–4, 6, 8, 9). These data suggest that G protein-coupled receptors can be modulated in a bidirectional manner, depending upon the interacting ligand.

The 5-HT_{2C} receptor belongs to the 5-HT_2 receptor subfamily of G protein-coupled receptors (10). Recently, the 5-HT_{2C} receptor was shown to exhibit constitutive activity in a transfected cell line (9). Three classes of 5-HT_{2C} receptor ligands were identified. Constitutive receptor activity was increased by agonists, decreased by inverse agonists, and not affected by a neutral antagonist (9). Consistent with properties opposite those of an agonist, binding of a radiolabeled inverse agonist was increased by the addition of guanine nucleotides. Furthermore, in a native system an agonist and an inverse agonist, but not a neutral antagonist, regulated 5-HT_{2C} receptor density (9).

The present study uses a heterologous expression system displaying constitutive 5-HT_{2C} receptor activity to evaluate additional 5-HT_{2C} receptor ligands in functional and radioligand binding assays. Several inverse agonists and a neutral antagonist were identified. Competition binding studies demonstrated that inverse agonists had higher affinity for the G protein-uncoupled form of the receptor, whereas agonists had higher affinity for the G protein-coupled form. Neutral antagonists bound both forms of the receptor with equal affinity. Thus, both the functional and binding properties of 5-HT_{2C} receptor inverse agonists were opposite those of agonists.

Experimental Procedures

Materials

NIH/3T3 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD). COS-6 cells were a generous gift from Dr. Lee Limbird, Vanderbilt University. D-MEM, CMRL-1066 medium, dialyzed calf serum, fetal bovine serum, penicillin, and streptomycin were purchased from GIBCO/BRL Life Technologies (Grand Island, NY). Calf serum was purchased from Hyclone Laboratories (Logan, UT). Cell culture dishes were purchased from Falcon/Becton Dickinson (Lincoln Park, NJ). Mianserin hydrochloride, pirenperone, and LY-

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); DOB, 1-(4-bromo-2,5-dimethoxyphenyl)-2-aminopropane; BOL, (+)-2-bromolysergic acid diethylamide tartrate; BES, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; PI, phosphoinositide; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; GDPβS, guanosine 5′-*O*-(2-thio)diphosphate; Gpp(NH)p, 5′-guanylylimidodiphosphate; D-MEM, Dulbecco's modified Eagle medium.

53,857 were purchased from Research Biochemicals (Natick, MA). Cyproheptadine hydrochloride and amitriptyline hydrochloride were purchased from Merck Sharp and Dohme Research Laboratories (West Point, PA). (−)-DOB and BOL were provided by the National Institute on Drug Abuse (Rockville, MD). *myo*-[^{3}H]Inositol (20–25 Ci/mmol) and (±)-[^{3}H]DOB (18.9 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA); [^{3}H]mesulergine (82 Ci/mmol) and [^{3}H] 5-HT (88 Ci/mmol) were purchased from Amersham Corporation (Arlington Heights, IL). BES was purchased from Calbiochem (La Jolla, CA). Pizotifen maleate, clozapine, and methysergide maleate were gifts from Sandoz (East Hanover, NJ). Metergoline was obtained from Farmatalia (Milan, Italy). Cinanserin hydrochloride was purchased from Squibb Institute for Medical Research (New Brunswick, NJ). 5-HT creatinine sulfate, prazosin hydrochloride, chlorpheniramine maleate, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Methods

Cell culture. NIH/3T3 fibroblasts stably transfected with 5-HT_{2C} receptor cDNA (11) and the selection marker pRSV/neo were isolated and characterized as described previously (9). A subclone stably expressing approximately 5 pmol of 5-HT_{2C} receptors/mg of protein was used in the present studies. NIH/3T3 fibroblasts were maintained in D-MEM supplemented with 10% calf serum, penicillin (5 units/ml), and streptomycin (5 μg/ml), in a humidified incubator at 5% CO_2 and 37°. COS-6 cells were maintained in D-MEM supplemented with 10% fetal bovine serum, penicillin (5 units/ml), and streptomycin (5 μg/ml). Transfection of COS-6 cells with the expression vector pCMV2/2C [5-HT_{2C} receptor cDNA subcloned into the *Eco*RI site of the pCMV2 (12) expression vector] was performed by calcium phosphate precipitation (13). Briefly, 2 × 10^6 COS-6 cells were plated and, 24 hr later, incubated with 9 ml of D-MEM containing 10% fetal bovine serum, penicillin, and streptomycin, along with the expression vector pCMV2/2C (10–20 μg/plate) in 1 ml of BBS buffer (140 mM NaCl, 750 μM Na_2HPO_4, 25 mM BES, 125 mM $CaCl_2$, pH 7). Eighteen hours later, cells were washed with phosphate-buffered saline and incubated with complete medium. Forty-eight hours later, cells were used in the binding assay.

PI hydrolysis assay. The accumulation of inositol monophosphate was assayed as described previously (9). Briefly, NIH/3T3 fibroblasts transfected with 5-HT_{2C} receptor cDNA were plated in 11-mm-diameter wells in D-MEM containing 10% calf serum, penicillin, and streptomycin. Once confluent, cells were labeled for 20–24 hr with 1 μCi of *myo*-[^{3}H]inositol/ml of CMRL-1066 medium. Lithium chloride (10 mM) and pargyline (10 μM) were added 5 min before the addition of the drugs. Incubations continued for 40 min. The reaction was terminated by aspiration of the medium and addition of methanol. [^{3}H] Inositol monophosphate was extracted with chloroform/methanol and isolated by anion exchange chromatography. Radioactivity was quantitated by liquid scintillation counting.

Radioligand binding. Radioligand binding was assayed in crude membrane homogenates prepared in 50 mM Tris·HCl, 10 mM $MgCl_2$, pH 7.5, as described previously (9). Briefly, 24 hr before assay, confluent monolayers of cells were changed into D-MEM without serum. On the day of assay, cells were washed with cold phosphate-buffered saline, scraped into tubes, and homogenized (Brinkmann Polytron homogenizer, setting 6, for 5 sec) in Tris·HCl/$MgCl_2$ buffer. Cell homogenates from NIH/3T3 fibroblasts transfected with 5-HT_{2C} receptor cDNA were subjected to one centrifugation at 20,000 × *g* for 30 min. The pellet was resuspended in Tris·HCl/$MgCl_2$ buffer and used in the binding assay. The centrifugation step was eliminated when COS-6 cells were assayed. In saturation binding experiments, [^{3}H]mesulergine or [^{3}H]5-HT was incubated for 30 min at 37° in a total volume of 600 μl. Nonspecific binding was defined with 10 μM methysergide or 10 μM mianserin, respectively. [^{3}H]5-HT binding was unaffected by the addition of pargyline; thus, pargyline was not routinely included in the binding buffer. Competition binding experiments were performed with 0.5 nM radioligand in the presence of increasing concentrations of nonlabeled competitor at 37° for 30 min. The effects of guanine nucleotides were assayed using a single concentration of radioligand in the presence of increasing concentrations of guanine nucleotide at 37° for 30 min. Samples were filtered using a Brandel harvester and Whatman GF/C filters that had been presoaked in 3% polyethylenimine, pH 9.5. Protein concentrations were determined by the method of Bradford (14), using bovine serum albumin as a standard.

Results

Identification of 5-HT_{2C} receptor inverse agonists. To evaluate their properties, 5-HT_{2C} receptor ligands were tested for the ability to decrease basal (agonist-independent) PI hydrolysis in NIH/3T3 fibroblasts transfected with 5-HT_{2C} receptor cDNA. A number of antagonists reduced basal PI hydrolysis with potencies consistent with interaction at the 5-HT_{2C} receptor (Table 1) and were classified as inverse agonists. As illustrated for clozapine and amitriptyline (Fig. 1), inverse agonists

TABLE 1

Potencies of inverse agonists to reduce constitutive activity of 5-HT_{2C} receptors expressed in fibroblasts

EC_{50} values were determined using the PI hydrolysis assay, as described in Experimental Procedures. All of these drugs produced 90–100% inhibition of basal activity. Values represent means ± standard errors of three or four individual experiments performed in triplicate.

Ligand	EC_{50}
	nM
Pizotifen	27 ± 7
Metergoline	38 ± 12
LY-53,857	45 ± 8
Methiothepin	48 ± 13
Clozapine	65 ± 6
Pirenperone	149 ± 6
Cyproheptadine	193 ± 34
Cinanserin	198 ± 30
Amitriptyline	280 ± 56

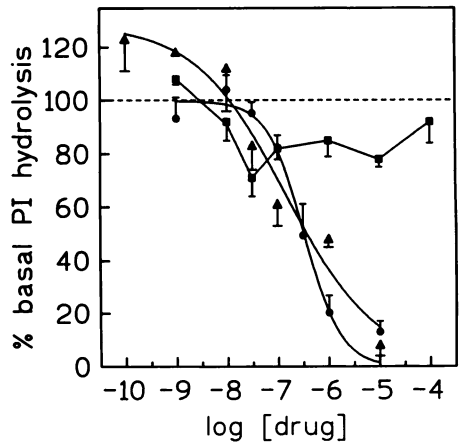

Fig. 1. 5-HT_{2C} receptor inverse agonists decrease, whereas a neutral antagonist has minimal effect on, basal PI hydrolysis in fibroblasts transfected with 5-HT_{2C} receptor cDNA. [^{3}H]Inositol-labeled cells were incubated with increasing concentrations of clozapine (▲), amitriptyline (●), or methysergide (■) for 45 min. [^{3}H]Inositol monophosphate was measured as described in Experimental Procedures. Data represent means ± standard errors of three separate experiments performed in triplicate. *Curves* were plotted using the equation for a sigmoid curve (InPlot; GraphPAD, San Diego, CA). Basal [^{3}H]inositol monophosphate formation (*horizontal dashed line*) ranged from 802 to 2105 cpm. EC_{50} values (mean ± standard error, three experiments) were 65 ± 6 nM for clozapine and 280 ± 56 nM for amitriptyline.

essentially eliminated basal PI hydrolysis. In contrast, methysergide had little effect on basal PI hydrolysis (Fig. 1), consistent with the properties of a neutral antagonist. In addition, the inverse agonists clozapine and amitriptyline had no effect on basal or sodium fluoride-stimulated PI hydrolysis in nontransfected NIH/3T3 fibroblasts (Fig. 2).

Guanine nucleotide modulation of [^{3}H]mesulergine and (±)-[^{3}H]DOB binding. Guanine nucleotide modulation of radiolabeled inverse agonist ([^{3}H]mesulergine) binding was evaluated to test the hypothesis that inverse agonists bind the G protein-coupled and -uncoupled forms of the 5-HT$_{2C}$ receptor with different affinities. As expected, guanine nucleotides decreased binding of the radiolabeled agonist (±)-[^{3}H]DOB in crude membranes prepared from NIH/3T3 fibroblasts transfected with 5-HT$_{2C}$ receptor cDNA (Fig. 3A). In contrast, [^{3}H] mesulergine binding increased with the addition of guanine nucleotides, but not ATP (Fig. 3A). In addition, GTPγS increased [^{3}H]mesulergine binding in COS-6 cells transiently transfected with 5-HT$_{2C}$ receptor cDNA (Fig. 3B). These data suggest that guanine nucleotides increased the affinity of [^{3}H] mesulergine for the 5-HT$_{2C}$ receptor and/or the density of binding sites. Saturation binding was performed to measure these parameters directly. In COS-6 cells transiently transfected with 5-HT$_{2C}$ receptor cDNA (Fig. 4), the affinity of [^{3}H] mesulergine was significantly higher in the presence (1.5 ± 0.4 nM) than in the absence (3.6 ± 0.8 nM) of GTPγS, without a significant change in the density of [^{3}H]mesulergine binding sites. A similar shift of [^{3}H]mesulergine affinity was observed in NIH/3T3 fibroblasts transfected with 5-HT$_{2C}$ receptor cDNA (data not shown). These data suggest that mesulergine had higher affinity for the G protein-uncoupled form than for the G protein-coupled form of the 5-HT$_{2C}$ receptor.

5-HT$_{2C}$ receptor ligands in competition binding. Competition binding was performed to determine whether other inverse agonists possessed different affinities for the G protein-coupled and -uncoupled forms of the 5-HT$_{2C}$ receptor. Competition binding studies frequently utilize an antagonist radioligand, which presumably labels the coupled and uncoupled forms of a receptor with equal affinity. This allows determination of the affinity of a competing ligand for each form of the receptor. However, in light of the evidence that [^{3}H]mesulergine binds the coupled and uncoupled forms of the 5-HT$_{2C}$ receptor with different affinities, the use of this ligand alone was not suitable. Instead, low concentrations of [^{3}H]5-HT and [^{3}H] mesulergine were used to independently label the coupled and uncoupled forms of the 5-HT$_{2C}$ receptor, respectively. The rationale for this design is based on the following results. Saturation binding demonstrated that [^{3}H]5-HT labeled a single population of receptors, with a K_d of 1.4 ± 0.2 nM (Fig. 5).

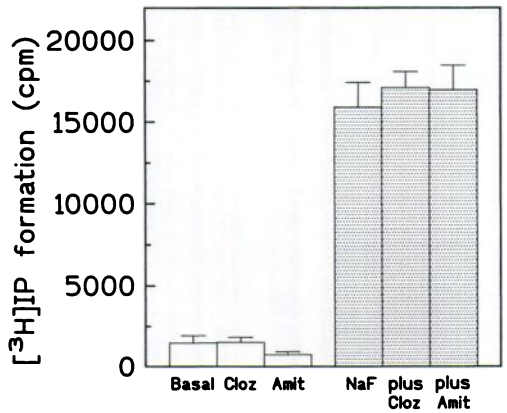

Fig. 2. 5-HT$_{2C}$ receptor inverse agonists have no effect on PI hydrolysis in nontransfected NIH/3T3 fibroblasts. [^{3}H]Inositol-labeled cells were incubated with vehicle (*Basal*) or 50 mM sodium fluoride (*NaF*) in the presence of vehicle, 1.3 μM clozapine (*Cloz*), or 5.6 μM amitriptyline (*Amit*). [^{3}H]Inositol monophosphate (*[^{3}H]IP*) was measured as described in Experimental Procedures. *Bars*, means ± standard errors of three separate experiments performed in triplicate. Sodium fluoride values versus corresponding basal levels yielded a significant effect ($p < 0.001$); all comparisons within each group were nonsignificant ($p > 0.05$). Data were analyzed using one-way analysis of variance, followed by Tukey-Kramer multiple-comparisons test (InStat; GraphPAD).

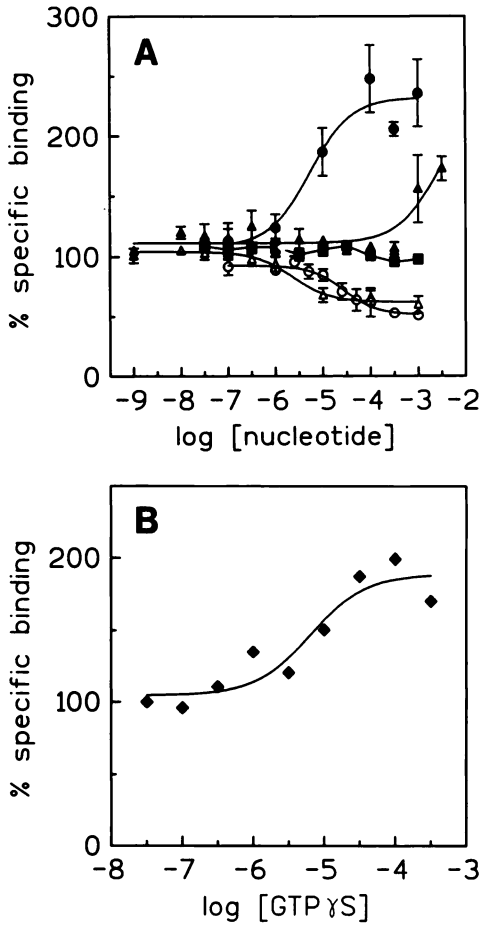

Fig. 3. Effects of nucleotides on agonist and inverse agonist radioligand binding. A, Membranes prepared from fibroblasts transfected with 5-HT$_{2C}$ receptor cDNA were incubated with GDPβS (*circles*), Gpp(NH)p (*triangles*), or ATP (*squares*), in the presence of 0.5 nM [^{3}H]mesulergine (*closed symbols*) or 5 nM (±)-[^{3}H]DOB (*open symbols*), as described in Experimental Procedures. Data represent means ± standard errors of four to six determinations from three individual experiments. EC$_{50}$ values (mean ± standard error, three experiments) for (±)-[^{3}H]DOB binding were 20 ± 3 μM for GDPβS and 2.9 ± 1 μM for Gpp(NH)p and those for [^{3}H] mesulergine binding were 9 ± 1 μM for GDPβS and 1.3 ± 0.4 mM for Gpp(NH)p. B, Whole-cell homogenates prepared from COS-6 cells transfected with 5-HT$_{2C}$ receptor cDNA were incubated with GTPγS and [^{3}H] mesulergine as described in Experimental Procedures. Data represent the means of duplicate determinations from a single experiment. Two additional experiments yielded similar curves with maximum increases in [^{3}H]mesulergine specific binding of 150% and 500%. The EC$_{50}$ value (mean ± standard error, three experiments) was 15.5 ± 8 μM. *Curves* were plotted using the equation for a sigmoid curve (InPlot; GraphPAD). Data are presented as percentage specific binding (the difference in radioligand bound in the presence and absence of 10 μM methysergide, normalized to specific binding in the absence of nucleotide).

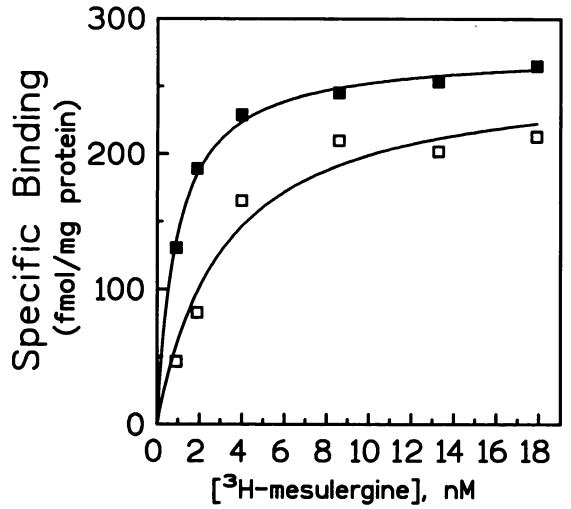

Fig. 4. GTPγS increases [^{3}H]mesulergine affinity for the 5-HT$_{2C}$ receptor. [^{3}H]Mesulergine (1–18 nM) saturation binding was performed in COS-6 cells transfected with 5-HT$_{2C}$ receptor cDNA, in the absence (□) or presence (■) of GTPγS (100 μM), as described in Experimental Procedures. Specific binding (the difference between [^{3}H]mesulergine bound in the absence and presence of 10 μM methysergide) is plotted as fmol of [^{3}H]mesulergine bound/mg of protein. Data are means of duplicate determinations and are representative of three separate experiments. *Curves* were plotted using the equation for a rectangular hyperbola (InPlot; GraphPAD). The K_d values (mean ± standard error) for control and GTPγS-treated preparations (four experiments) were 3.6 ± 0.8 and 1.5 ± 0.4 nM, respectively ($p = 0.02$). The B_{max} values (mean ± standard error) for control and GTPγS-treated preparations (four experiments) were 1199 ± 775 and 1197 ± 785 fmol of [^{3}H]mesulergine bound/mg of protein, respectively ($p > 0.05$). Data were analyzed using a paired Student *t* test.

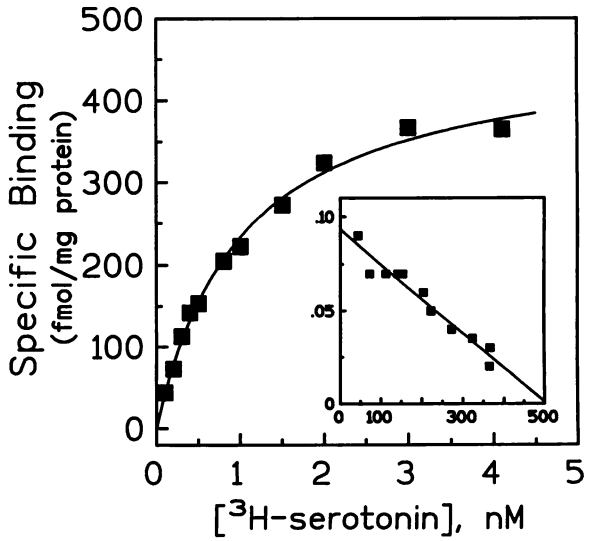

Fig. 5. [^{3}H]5-HT binds a single class of 5-HT$_{2C}$ receptors with high affinity in fibroblasts transfected with 5-HT$_{2C}$ receptor cDNA. Saturation binding (0.1–4 nM) was performed in membranes prepared from fibroblasts transfected with 5-HT$_{2C}$ receptor cDNA, as described in Experimental Procedures. Specific binding (the difference between [^{3}H]5-HT bound in the absence and presence of 10 μM mianserin) is plotted as fmol of [^{3}H] 5-HT bound/mg of protein. Data are means of duplicate determinations and are representative of three separate experiments. The K_d was 1.4 ± 0.2 nM (mean ± standard error, three experiments). *Curves* were plotted using the equation for a rectangular hyperbola (InPlot; GraphPAD). *Inset*, saturation binding data were transformed to a Scatchard plot. *Ordinate*, bound ligand/free ligand; *abscissa*, bound ligand (fmol of [^{3}H]5-HT bound/mg of protein).

In addition, GTPγS eliminated specific binding of 1 nM [^{3}H]5-HT (data not shown). Thus, at a concentration of 0.5 nM, [^{3}H] 5-HT labeled the coupled form of the 5-HT$_{2C}$ receptor. In competition binding, the affinity of mesulergine for the [^{3}H]5-HT-labeled site was 6 nM (Table 2), whereas [^{3}H]mesulergine saturation binding in the presence of GTPγS yielded a K_d of 1.8 ± 0.3 nM (Table 2). Thus, at a concentration of 0.5 nM [^{3}H] mesulergine, >90% of the specific binding reflected the uncoupled form of the receptor. IC_{50} values obtained from competition binding at the coupled ([^{3}H]5-HT-labeled) and uncoupled ([^{3}H] mesulergine-labeled) forms of the 5-HT$_{2C}$ receptor were converted to K_i values using the Cheng-Prusoff equation (15).

Consistent with classical models of agonist-receptor interaction, (−)-DOB (Fig. 6A) had higher affinity for the [^{3}H]5-HT-labeled form of the receptor. In contrast, the inverse agonists clozapine (Fig. 6B), ketanserin, and cyproheptadine (Table 2) had higher affinity for the uncoupled ([^{3}H]mesulergine-labeled) form of the receptor. The neutral antagonists BOL (Fig. 6C) and methysergide (Table 2) had equal affinity for both forms of the 5-HT$_{2C}$ receptor.

TABLE 2

Differential binding affinities of agonists, inverse agonists, and neutral antagonists

IC_{50} values were obtained from competition binding assays, as described in Experimental Procedures. K_i values (mean ± standard error; three experiments) were determined by conversion of IC_{50} values using the Cheng-Prusoff equation.

Ligand	K_i		*p* value[a]
	[^{3}H]5-HT	[^{3}H]Mesulergine	
	nM		
Agonists			
(−)-DOB	1 ± 0.1	121 ± 13	0.0008
5-HT	1.4 ± 0.2[b]	378 ± 35	0.0003
Inverse agonists			
Clozapine	213 ± 34	25 ± 7	0.0056
Cyproheptadine	49 ± 5	18 ± 3	0.0064
Ketanserin	372 ± 46	130 ± 28	0.01
Mesulergine	6 ± 1.6	1.8 ± 0.3[c]	0.0274
Neutral antagonists			
BOL	8.5 ± 0.7	7.9 ± 0.1	NS[d]
Methysergide	1.5 ± 0.1	2 ± 0.3	NS

[a] Based on unpaired Student's *t* test.
[b] Determined by [^{3}H]5-HT saturation binding.
[c] Determined by [^{3}H]mesulergine saturation binding.
[d] NS, not statistically significant ($p > 0.05$).

Discussion

The 5-HT$_{2C}$ receptor is implicated in a variety of disease processes, including anxiety (16), schizophrenia (17), and affective disorders (18). Hallucinogenic compounds also potently interact with the 5-HT$_{2C}$ receptor (19). Heterologous expression of the cloned 5-HT$_{2C}$ receptor in fibroblasts results in constitutive receptor activation (9). Some 5-HT$_{2C}$ receptor ligands (termed inverse agonists) decrease constitutive activity and block receptor activation by agonists. The neutral antagonist BOL has no effect on constitutive activity but blocks the effects of an agonist and an inverse agonist, consistent with the idea that the effects of the different drugs are mediated through the 5-HT$_{2C}$ receptor (9). The present studies identified additional 5-HT$_{2C}$ receptor inverse agonists with markedly different chemical structures and distinct clinical profiles. For example, the atypical antipsychotic drug clozapine and the tricyclic antidepressant amitriptyline exhibited negative intrinsic activity at

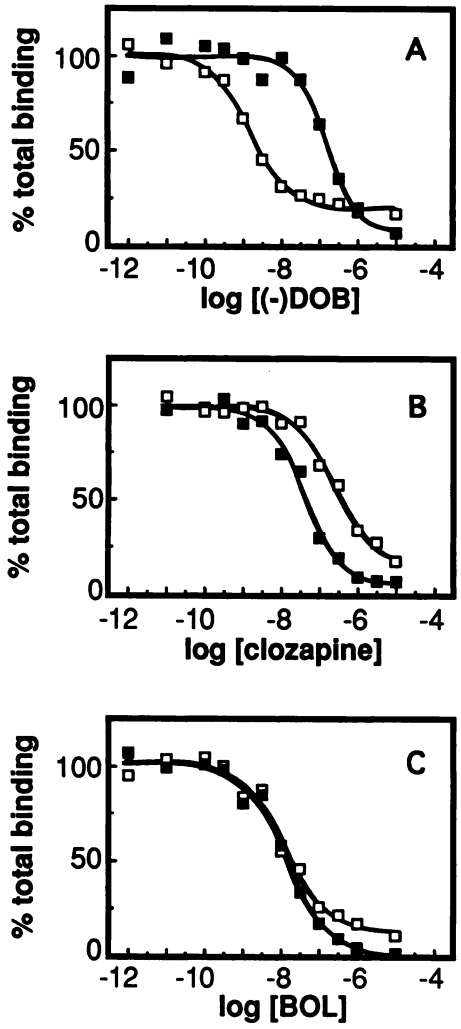

Fig. 6. 5-HT$_{2C}$ receptor ligand bind differentially at [^{3}H]5-HT and [^{3}H] mesulergine sites in fibroblasts transfected with 5-HT$_{2C}$ receptor cDNA. Competition binding was performed as described in Experimental Procedures, using 0.5 nM [^{3}H]5-HT (□) or [^{3}H]mesulergine (■). A, 5-HT$_{2C}$ receptor agonist (−)-DOB; B, 5-HT$_{2C}$ receptor inverse agonist clozapine; C, neutral antagonist BOL. Values are means of duplicate determinations and are representative of three individual experiments. *Curves* were plotted using the equation for a sigmoid curve (InPlot; GraphPAD). See Table 2 for a summary of all data.

the 5-HT$_{2C}$ receptor. These inverse agonists had no effect on basal or sodium fluoride-stimulated PI hydrolysis in nontransfected fibroblasts. Thus, the effects of inverse agonists reflect an interaction with the 5-HT$_{2C}$ receptor. The neutral antagonists identified thus far (BOL and methysergide) are ergot derivatives. Other ergots act as agonists (e.g., lysergic acid diethylamide)[1] or inverse agonists (e.g., metergoline, mesulergine, and LY-53,857).

Guanine nucleotides promote the dissociation of receptor-G protein complexes and provide a tool to discriminate multiple agonist affinity states of G protein-coupled receptors. In addition, guanine nucleotides increase radiolabeled antagonist binding in a variety of systems (20–26). To allow detection of different receptor affinity states, binding assays are routinely performed in the absence of sodium. This is due to the fact that sodium promotes dissociation of the receptor-G protein complex, thereby eliminating the ability to detect modulation of this interaction by guanine nucleotides. The present study did not address the effects of sodium on radioligand binding. However, our demonstration that guanine nucleotides modulated agonist and inverse agonist binding under the conditions described is consistent with previous studies. Even though the present study demonstrated that guanine nucleotides increased the affinity of [^{3}H]mesulergine for the 5-HT$_{2C}$ receptor, this is not always observed. For example, Havlik and Peroutka (27) reported that GTP does not alter [^{3}H]mesulergine binding to the 5-HT$_{2C}$ receptor. This negative result may have been due to the use of submaximal concentrations of GTP. We found GTP to be a weak modulator of [^{3}H]mesulergine binding, requiring concentrations of >1 mM. In addition, the degree of modulation of [^{3}H]mesulergine binding may depend on the level of basal receptor activation or the stoichiometry between receptors and G proteins, parameters that are not readily controlled.

The increase in inverse agonist binding produced by guanine nucleotides is consistent with the interpretation that inverse agonists possess higher affinity for the uncoupled form of the 5-HT$_{2C}$ receptor. To measure directly the affinity of ligands for the two forms of the 5-HT$_{2C}$ receptor, assay conditions were established to separately label the G protein-coupled and -uncoupled forms. The differential affinities of agonists, inverse agonists, and neutral antagonists were then evaluated. As expected, agonists had higher affinity for the G protein-coupled form of the receptor, thus validating the use of this experimental design. All of the inverse agonists exhibited higher affinity for the uncoupled form of the receptor, consistent with the findings of guanine nucleotide modulation of radiolabeled inverse agonist binding. Importantly, the neutral antagonists had equal affinity for both forms of the receptor. Thus, the relative affinities of 5-HT$_{2C}$ receptor ligands for the coupled and uncoupled forms of the receptor are consistent with the functional classification of the ligands, as well as with the reciprocal guanine nucleotide modulation of radiolabeled agonist and inverse agonist binding.

The working hypothesis regarding constitutive 5-HT$_{2C}$ receptor activity is that a significant proportion of receptors couple to G proteins in the absence of agonist. Our data suggest that 5-HT$_{2C}$ receptor inverse agonists decrease basal receptor activation by binding with higher affinity to the uncoupled form of the receptor, shifting receptor equilibrium away from the precoupled state. Agonists, on the other hand, increase receptor activation by binding with higher affinity to the receptor in a conformation that interacts with G proteins. Neutral antagonists bind both forms of the receptor with equal affinity and therefore have no effect on basal receptor activity but block the effects of both agonists and inverse agonists. This model is consistent with other recent models of G protein-coupled receptor activation (7, 28, 29).

Constitutive activation of 5-HT$_{2C}$ receptors in a heterologous expression system has revealed novel properties of 5-HT$_{2C}$ receptor antagonists. Importantly, the distinction of inverse agonists and neutral antagonists has carried over to native systems of 5-HT$_{2C}$ receptor expression. Previous studies in our laboratory have shown that an agonist and an inverse agonist, but not a neutral antagonist, decrease 5-HT$_{2C}$ receptor density in primary cultures of choroid plexus epithelial cells that natively express the 5-HT$_{2C}$ receptor (9). Thus far, lack of assay

[1] Unpublished observations.

sensitivity has precluded delineation of inverse agonist effects on PI hydrolysis using this system. *In vivo* studies have demonstrated that rats trained in a 5-HT_{2A} receptor agonist-inverse agonist discrimination paradigm distinguish the effects of agonists and inverse agonists from those of a neutral antagonist.[2] These studies suggest that functionally distinct properties of inverse agonists and neutral antagonists extend beyond the heterologous expression system.

5-HT_{2C} receptor inverse agonists possess properties opposite the properties of agonists with respect to receptor activation and receptor binding. It is of interest to identify additional properties unique to inverse agonists and to determine whether inverse agonists alter post-translational modifications of the receptor, such as the phosphorylation state. In addition, future experiments should address the functional cellular consequences of ligand modulation of constitutive 5-HT_{2C} receptor activity.

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[2] [R. S. Smith, R. Barrett, and E. Sanders-Bush, unpublished observations.

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