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Serotonergic Antagonists Differentially Inhibit Spontaneous Activity and Decrease Ligand Binding Capacity of the Rat 5-Hydroxytryptamine Type 2C Receptor in Sf9 Cells

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SUMMARY

The activities of serotonergic antagonists as inverse agonists at the rat 5-hydroxytryptamine (5-HT)$_{2C}$ serotonin receptor were compared with their potencies in promoting receptor "down-regulation," after expression of the recombinant receptor in the baculovirus/Sf9 insect cell system. Baculovirus expression yielded high levels of 5-HT$_{2C}$ receptors (up to 10^6 receptors/cell), which were functionally coupled to polyphosphoinositide turnover in Sf9 cells through a pertussis toxin-insensitive pathway. The expressed receptor exhibited spontaneous activation of inositol phosphate production, which was inhibited in a dose-dependent manner by serotonergic antagonists, consistent with inverse agonist activity. The potencies of antagonists as inverse agonists correlated with their respective binding affinities determined in competition binding studies with membrane preparations. The maximal inhibition of spontaneous activity ranged from 32% inhibition for mianserin to no effect for spiroxatrine, indicating that antagonists differ in their intrinsic inverse efficacies. Antagonist treatment of intact Sf9 cells or membranes containing the 5-HT$_{2C}$ receptor, followed by washout of residual drug, resulted in a decrease (up to 90%) in the number of binding sites for [^{3}H]mesulergine and [^{3}H]5-HT, with no change in the affinity for [^{3}H]mesulergine. The decrease in binding was irreversible, was not due to the presence of residual antagonist, and was not observed after treatment with agonists. This effect of antagonists in membranes was dose dependent, but the rank order of potency was clearly different from that for inverse agonist activity, indicating that the two effects reflect distinct actions of antagonists at the 5-HT$_{2C}$ receptor. The relative abilities of antagonists to produce loss of binding showed a good correlation with their reported abilities to down-regulate 5-HT$_2$ receptors *in vivo* after chronic treatment, suggesting that these actions reflect the same underlying process.

Serotonin (5-HT) acts as a neurotransmitter and circulating hormone to control a wide variety of physiological processes. The diverse functions of 5-HT are mediated by multiple 5-HT receptor subtypes (1). With the exception of the 5-HT$_3$ receptors, which are ligand-gated ion channels, the serotonin receptors are members of the G protein-coupled receptor superfamily and function via trimeric G proteins to modulate the activities of various cellular effectors, including adenylyl cyclase and phopholipase C (2).

The 5-HT$_2$ receptors include three subtypes (5-HT$_{2A}$, 5-HT$_{2B}$, and 5-HT$_{2C}$), which activate polyphosphoinositide turnover via a pertussis toxin-insensitive G protein. The 5-HT$_{2A}$ and 5-HT$_{2C}$ subtypes are widely distributed in brain, with the 5-HT$_{2C}$ receptor (formerly named 5-HT$_{1C}$) being particularly abundant in choroid plexus, from which the corresponding cDNA was originally cloned (3). Although the physiological roles of the 5-HT$_2$ receptors are poorly understood, the pharmacology of the 5-HT$_{2A}$ and 5-HT$_{2C}$ subtypes has received considerable attention due to the demonstrated therapeutic efficacy of selective antagonists as antidepressant and antipsychotic agents (4, 5).

A notable feature of antagonist action at the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors is that certain antagonists, when administered chronically to rats, produce a decrease in receptor levels in various brain regions, as measured by radioligand binding. This loss of binding upon prolonged antagonist treatment has been termed "atypical down-regulation," reflecting the general view that agonists normally down-regulate, whereas antagonists up-regulate, receptor numbers. It has been proposed that this atypical action of antagonists represents an adaptive response that could underlie the therapeutic efficacy of serotonergic antagonists (4, 5). The mechanism by which antagonists exert this effect is unclear.

A recent study provided evidence that serotonergic antag-

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; IP, inositol phosphate; DOI, 2,5-dimethoxy-4-iodoamphetamine hydrobromide; PBS, phosphate-buffered saline.

onists exhibit inverse agonist activity at the recombinant 5-HT_{2C} receptor expressed in a mammalian cell line (6, 7). The ability of antagonists to inhibit spontaneous, agonist-independent receptor activity has been observed for numerous G protein-coupled receptors (8–13) and may represent a general property of antagonist action on members of this receptor family. Comparison of the effects of two antagonists on spontaneous activity of the recombinant 5-HT_{2C} receptor with their abilities to reduce receptor numbers in primary cultures of choroid plexus led to the proposal (6) that inverse agonist activity was directly correlated with atypical down-regulation of 5-HT_{2C} receptors by antagonists *in vivo*.

In an effort to further investigate the possible relationship between inverse agonism and atypical down-regulation, we have analyzed the effects of serotonergic antagonists on spontaneous activity and receptor numbers for the rat 5-HT_{2C} receptor expressed in the baculovirus/insect cell system. The results show that the baculovirus-expressed 5-HT_{2C} receptor mediates agonist-stimulated release of IPs in intact Sf9 cells and exhibits elevated spontaneous activity, which is inhibited by antagonists possessing inverse agonist activity. Furthermore, pretreatment of intact cells or membrane preparations with antagonists, but not with agonists, resulted in a dose-dependent decrease in the binding capacity for [^{3}H]mesulergine and [^{3}H]5-HT. The rank order of potency for this effect was clearly different from that for inverse agonist activity but showed a striking correlation with the reported abilities of the antagonists to promote atypical down-regulation of 5-HT_{2C} receptors *in vivo*. Comparison of these two effects of antagonists on the baculovirus-expressed 5-HT_{2C} receptor clearly indicates that inverse agonist activity and the ability to promote loss of receptor binding capacity represent distinct actions of antagonists and that drugs can selectively target one or the other action. Part of this work has been presented previously (14).

Materials and Methods

Reagents. Buffer chemicals and protease inhibitors were purchased from Sigma, and cell culture media were from Gibco/BRL. Unlabeled ligands were supplied by Research Biochemicals International, with the exception of RU24969, which was a gift from Roussel Uclaf. [^{3}H]Mesulergine (78–82 Ci/mmol) was purchased from Amersham and *myo*-[^{3}H]inositol (10–20 Ci/mmol) was purchased from NEN-DuPont. The AG 1X-8 ion exchange resin was supplied by Bio-Rad.

The recombinant baculovirus used for expression of the rat 5-HT_{2C} receptor was provided by the Biotechnology Research Institute of Montreal. The virus was constructed using a synthetic DNA fragment encoding the rat 5-HT_{2C} receptor, based on the published sequence of the cloned cDNA from choroid plexus (3). The synthetic cDNA was prepared and its sequence was verified by Allelix Biopharmaceuticals. The cDNA was inserted into the IpDC-126 baculovirus transfer vector, and a recombinant baculovirus was produced and purified as described previously (15).

Cell culture and receptor expression. Sf9 cells were cultured in 50-ml batches in 250-ml shaker flasks at 27°, in Sf-900 II serum-free medium containing 50 μg/ml gentamicin sulfate. Cells were grown to a density of 3×10^6 cells/ml and infected with the 5-HT_{2C} recombinant baculovirus or with wild-type *Autographia californica* nuclear polyhedrosis baculovirus, at a multiplicity of infection of 2. Viral stocks for infections were in Grace's insect medium containing 5% fetal bovine serum (Hyclone) and were added to cultures at a dilution of approximately 1/20 upon infection. The infected cells were maintained in culture for various periods and used for analysis of ligand binding and measurement of IP production as described below.

Radioligand binding assays. For the estimation of total receptor numbers in intact Sf9 cells, the cells were pelleted by low-speed centrifugation (3 min at 800 rpm in a Sorvall H6000A rotor), followed by resuspension in PBS and recentrifugation, and were resuspended in either PBS or binding buffer (50 mM Tris·HCl, pH 7.4, 15 mM $MgCl_2$, 2 mM EDTA, 0.1% ascorbic acid, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 20 μg/ml benzamidine, 50 μg/ml *n*-tosyl-L-phenylalanine chloromethyl ketone, 50 μg/ml trypsin inhibitor). Cell viability after washing was estimated at 60–70% by trypan blue exclusion. Aliquots of 10,000 cells were incubated for 1 hr at 27°, in a final volume of 540 μl containing 20 nM [^{3}H]mesulergine. Incubations were terminated by vacuum filtration over GF/C filters and washing with binding buffer at 4°. Bound radioactivity was measured on filters impregnated with MeltiLex melt-on scintillant, using a Wallac MicroBeta counter. Nonspecific binding was estimated in parallel incubations containing 10 μM mianserin or metergoline.

For analysis of ligand binding to membrane preparations, cultures were harvested 48 hr after infection and lysed, and a membrane pellet was prepared as described previously (16) and stored at −80°. Protein concentrations were determined by the nitrocellulose amido black method (17). Membranes were thawed on ice and resuspended in binding buffer by homogenization with a Potter homogenizer. The membranes (5 μg) were incubated with [^{3}H]mesulergine for 1 hr at 27°, in a final volume of 540 μl, and the assays were terminated as described above for intact cells. Saturation binding assays yielded a K_d for [^{3}H]mesulergine of 2 nM, and competition binding assays with unlabeled drugs were carried out with 3 nM [^{3}H]mesulergine (Table 1).

Analysis of binding kinetics. Time course association experiments with [^{3}H]mesulergine were carried out by measuring [^{3}H]mesulergine bound at different times after addition of radioligand, as described above (also see the figure legends). Dissociation experiments were performed by incubating membranes for 60 min at 27° with [^{3}H]mesulergine at a concentration of 20 nM or 500 nM (see Results). The labeled membranes were then centrifuged (2 min at 12,000 rpm in a microfuge) and resuspended in a 1000-fold excess of either binding buffer alone or buffer containing unlabeled drugs (see Results). The diluted membranes were then incubated at 27°, and aliquots were collected in triplicate (with a 30-ml syringe) and vacuum filtered over 24-mm GF/C filters, using a Millipore 1225 sampling manifold. Bound radioactivity was measured on the filters by scintillation counting in 1 ml of Hi-load LKB scintillation cocktail. Control membranes were processed in parallel to assess stability.

IP production. Growing cell cultures (1×10^6 cells/ml) were prelabeled with 1 μCi/ml *myo*-[^{3}H]inositol for 24 hr before infection, and the labeled cells were then transferred to 50-ml shaker flasks for infection. Assays (two or three replications, as indicated) were started by addition of labeled cells ($2.5–10 \times 10^5$ cells/well) to a 96-well, deep-well plate (Beckman) containing the drugs to be tested, followed by immediate mixing. The cells were then incubated for 20 min at 27° in a shaking incubator, and the incubations were stopped by addition of perchloric acid. Total ^{3}H-labeled IPs were measured by scintillation counting after isolation by anion exchange chromatography on AG 1X-8 resin, as described previously (18).

Effect of drug pretreatment on [^{3}H]mesulergine binding. Infected Sf9 cell cultures were treated with antagonists by addition of drugs directly to the culture medium 12 hr before cell harvest. Cells from cultures treated in triplicate were then quickly washed with culture medium to remove unbound ligands. Before measurement of total [^{3}H]mesulergine binding, dissociation of bound ligand was induced by dilution of the cells (1/500) in PBS/Grace buffer (PBS supplemented with 26 g/liter sucrose, 0.7 g/liter dextrose, 4.1 g/liter KCl, 1.36 g/liter $MgSO_4$, 1.01 g/liter $MgCl_2$) and washing of the cells in the same buffer for three 60-min cycles at 27°. Cells were then resuspended in binding buffer, and receptor levels were determined as described above, with 10 nM [^{3}H]mesulergine.

Drug pretreatments of membranes were initiated by transfer of 500 μl of membranes in binding buffer (i.e., 200 μg/assay for each replicate of each drug treatment) to polypropylene tubes (containing 5-HT ligands) and incubation for 60 min at 27°. Treatments were stopped by centrifugation of membranes at 4000 rpm for 10 min in a Sorvall H6000A rotor. Membranes were extensively washed as described above for whole cells and were resuspended in binding buffer (10 μg/assay), and the total binding and nonspecific binding of 10 nM [^{3}H]mesulergine were measured in triplicate.

Analysis of data. The binding of [^{3}H]mesulergine (saturation experiments) and the inhibition of [^{3}H]mesulergine binding by unlabeled serotonergic ligands were analyzed in terms of a single class of binding sites, using the computer program LIGAND (19). Data from three independent binding experiments were fitted individually, and the affinities presented for [^{3}H]mesulergine (K_d) and other ligands (K_i) represent average ± standard error values from three sets of data. Dose-response data for IPs and antagonist-induced decreases in [^{3}H]mesulergine binding capacity were scaled by taking values measured in the absence of added ligand as 100%. The scaled data were analyzed according to a four-parameter logistic equation analogous to the Hill equation (ALLFIT from A. DeLean, Department of Pharmacology, Université de Montréal, or INPLOT from GraphPad Software, San Diego, CA). For the decrease in [^{3}H]mesulergine binding with each ligand, three or four sets of data were fitted simultaneously using ALLFIT, with the slope factor being set equal to 1. Additional details are described in the table and figure legends. Maximal inverse agonist activities measured for each drug tested were compared statistically by a two-tailed t test ($\alpha = 0.05$).

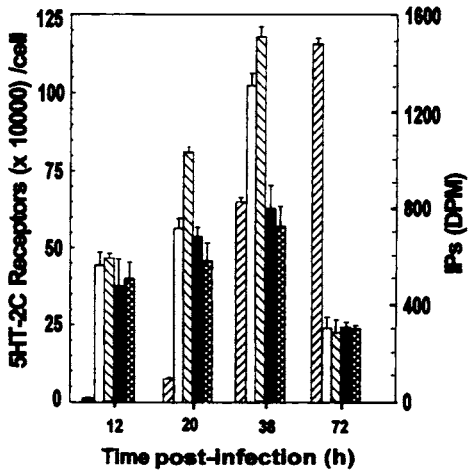

Fig. 1. Functional coupling of the rat 5-HT$_{2C}$ receptor to endogenous phospholipase C in Sf9 cells. Sf9 cells labeled with *myo*-[^{3}H]inositol (1 μCi/ml) were infected with a baculovirus encoding the 5-HT$_{2C}$ receptor. [^{3}H]Mesulergine binding was measured in whole cells at 12 hr, 20 hr, 38 hr, and 72 hr after infection (▨). IP levels were measured in cells that had been incubated for 20 min in medium (Sf-900 II medium with 20 mM LiCl) without the addition of drugs (□), with 1 μM 5-HT (▧), with both 1 μM 5-HT and 10 μM mianserin (■), or with 10 μM mianserin alone (■). In cells infected with wild-type virus, IP levels were not affected by serotonergic drugs and no specific [^{3}H]mesulergine binding was detected (data not shown).

Results

Expression of functional 5-HT$_{2C}$ receptors in Sf9 insect cells. A baculovirus encoding the rat 5-HT$_{2C}$ receptor was used to express the recombinant receptor in cultures of Sf9 insect cells. Receptor levels, as measured in whole cells by the binding of [^{3}H]mesulergine (10 nM), increased with time after infection to reach approximately 1×10^6 sites/cell at 72 hr after infection (Fig. 1). The ability of the expressed 5-HT$_{2C}$ receptor to modulate endogenous phospholipase C activity in intact Sf9 cells was assessed by measuring the production of total IPs over 20 min, as described in Materials and Methods. The levels of intracellular IPs at different times after infection and with various pharmacological treatments are presented in Fig. 1. The basal level of IP production increased over the time course of the infection and at 38 hr after infection was roughly double that at 20 hr. Basal IP production dramatically decreased at 72 hr, reaching levels comparable to those observed for cells infected with wild-type baculovirus (data not shown). This loss of activity at 72 hr, the point of highest receptor expression, paralleled a drop in cell viability (to <10%), consistent with a generalized loss of cell function at late stages of viral infection.

As shown in Fig. 1, the natural agonist 5-HT was able to stimulate IP production in whole cells expressing 5-HT$_{2C}$ receptors; the response to 5-HT, however, varied over the course of the infection. At 20 hr and 38 hr after infection, 5-HT stimulated IP production by 30% and 13%, respectively, over basal activity measured at the same time point. The response to 5-HT was completely inhibited by the antagonist mianserin (10 μM). The agonist had no effect on basal IP release at 12 hr after infection, when there was little or no receptor expression (Fig. 1), or in cells infected with wild-type virus (data not shown). The response to 5-HT was lost at 72 hr, in parallel with the drop in basal activity.

TABLE 1

Pharmacology of the 5-HT$_{2C}$ receptor expressed in Sf9 cells

Results are expressed as the mean ± standard error for at least three experiments, and maximal activity represents the percentage of IP inhibition at the highest drug concentration (from Fig. 3). Antagonist binding affinities were measured in membranes from Sf9 cells expressing 5-HT$_{2C}$ receptors (48 hr after infection) and IP responses were measured in whole cells (38 hr after infection), as described in Materials and Methods.

Antagonists	Binding, pK_i	IP responses		
		pEC$_{50}$	Hill coefficient	Maximal activity
				% inhibition
Metergoline	9.22 ± 0.10	8.81 ± 0.20	0.7 ± 0.2	26.3 ± 3.5
Methysergide	9.12 ± 0.05	7.95 ± 0.36	0.9 ± 0.2	21.1 ± 4.0
Ritanserin	8.89 ± 0.10	7.75 ± 0.36	0.8 ± 0.2	23.4 ± 4.3
Mianserin	8.63 ± 0.05	7.45 ± 0.24	0.9 ± 0.2	32.2 ± 2.6
Mesulergine	8.70 ± 0.05	7.35 ± 0.46	0.5 ± 0.2	17.6 ± 3.6
Ketanserin	7.69 ± 0.02	6.70 ± 0.21	1.2 ± 0.4	16.0 ± 3.6
Clozapine	7.62 ± 0.20	6.76 ± 0.36	1.0 ± 0.1	20.0 ± 2.0
Spiperone	5.92 ± 0.10	5.08 ± 0.41	1.2 ± 0.3	20.0 ± 2.7
Spiroxatrine[a]	5.17 ± 0.08	5.04 ± 0.99		−2.0 ± 2.0

[a] Spiroxatrine does not affect polyphosphoinositide turnover (Fig. 3) but competes with 5-HT activation (pIC$_{50}$ = 5.04) (Fig. 5).

The concentration dependence of the stimulation of IP production by the serotonergic agonists 5-HT, DOI, and RU24959 was studied in whole cells at 20 hr after infection (Fig. 2). The rank order of potency for the agonists was 5-HT = DOI > RU24959 (see the legend to Fig. 2). Whereas DOI and 5-HT stimulated IP production to equal extents (approximately 30% stimulation), RU24959 acted as a partial agonist of the response (12% stimulation). Treatment of the cells with pertussis toxin (up to 100 ng/ml) for 24 hr before measurement of IP production did not alter the stimulatory effect of 5-HT (data not shown).

The antagonist mianserin, in addition to blocking the stimulatory effect of 5-HT, also produced an inhibition of basal IP production in the absence of added agonist. The extent of this inhibition varied from 20% at 20 hr after infection to roughly 40% inhibition at 38 hr. Mianserin had no effect on basal levels of IP production at 12 hr or 72 hr after infection. These results suggest that the baculovirus-expressed 5-HT$_{2C}$ receptor exhibits spontaneous, agonist-independent activity, which can be inhibited by the antagonist mianserin. Mianserin thus appears to act as an inverse agonist at the 5-HT$_{2C}$ receptor, in agreement with a previous report (6).

Analysis of inverse agonist activity. The pharmacology of a series of serotonergic antagonists at the 5-HT$_{2C}$ receptor in Sf9 cells was examined in binding studies using membranes prepared from Sf9 cells at 48 hr after infection. Saturation binding experiments with these membranes and [^{3}H]mesulergine gave a receptor concentration of 27 pmol/mg of protein and an affinity (K_d) of 2 nM. The binding affinities for the antagonists (Table 1), as determined in competition binding assays, showed the following rank order: metergoline > methysergide > ritanserin > mesulergine = mianserin > ketanserin = clozapine > spiperone > spiroxatrine. All of the antagonists appeared to bind to a single class of sites (the Hill coefficients were indistinguishable from 1). These results are in good agreement with those reported for the binding of antagonists to rat 5-HT$_{2C}$ receptors present in choroid plexus (see Ref. 20 for review) or expressed in mammalian cells (6, 21–23).

The effects of the antagonists on basal IP production, analyzed in whole cells at 38 hr after infection, are shown in Fig. 3. All of the antagonists tested, with the exception of spiroxatrine, produced a dose-dependent inhibition of IP production. The EC$_{50}$ values for inhibition by the various antagonists (Table 1) showed the same rank order as the binding affinities, and an excellent correlation (r^2 = 0.97) was observed between the two parameters (Fig. 4). The maximal inhibition varied depending on the antagonist, ranging from 32% for mianserin to no effect for spiroxatrine at concentrations up to 500 μM (100 times its binding affinity). The rank order for maximal inhibition was as follows: mianserin > metergoline ≥ ritanserin ≥ methysergide ≥ clozapine ≥ spiperone ≥ mesulergine ≥ ketanserin. This clearly differs from the rank order observed for inhibitory potency and binding affinity for this series of antagonists. These findings

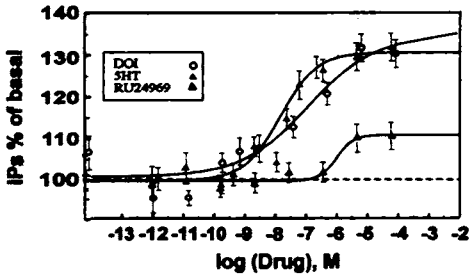

Fig. 2. Effects of serotonergic agonists on IP release in 5-HT$_{2C}$ receptor-expressing Sf9 cells. Sf9 cells labeled with *myo*-[^{3}H]inositol (1 μCi/ml) were infected with a baculovirus encoding the 5-HT$_{2C}$ receptor, and IP levels were measured in whole cells at the agonist concentrations indicated (*abscissa*). Data represent averaged values for the percentage of IP levels, with basal levels being taken as 100%, for DOI, 5-HT, and RU24959. *Curves* were fitted and generated using INPLOT, as described in Materials and Methods. The EC$_{50}$ estimates and corresponding slope factors were as follows: 5-HT, 0.02 ± 0.01 μM and 1.2; DOI, 0.1 ± 0.05 μM and 0.7; RU24959, 1.3 ± 0.1 μM and 1.3, respectively.

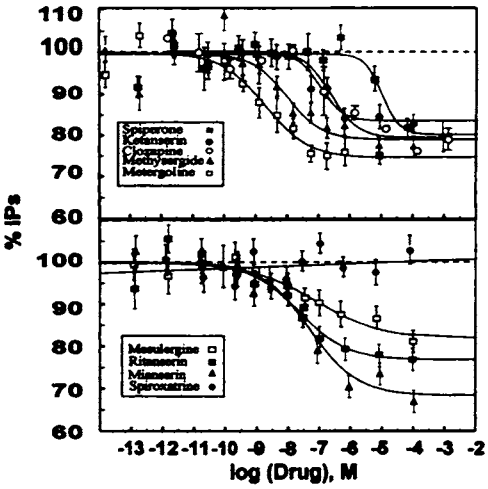

Fig. 3. Effects of serotonergic antagonists on IP release in 5-HT$_{2C}$ receptor-expressing Sf9 cells. Sf9 cells labeled with *myo*-[^{3}H]inositol were infected with the 5-HT$_{2C}$ receptor baculovirus, and IPs were measured at 38 hr after infection in whole cells, in the presence of various serotonergic antagonists. Data from three independent assays were sealed, taking basal levels of IP release (i.e., in the absence of any serotonergic ligands) as 100%. The points shown (± standard error) represent the averaged scaled data. EC$_{50}$ estimates are reported in Table 1 with the corresponding slope factors (Hill coefficients). Spiroxatrine had no effect on basal IP levels at 20 hr, 38 hr, or 48 hr after infection (data not shown).

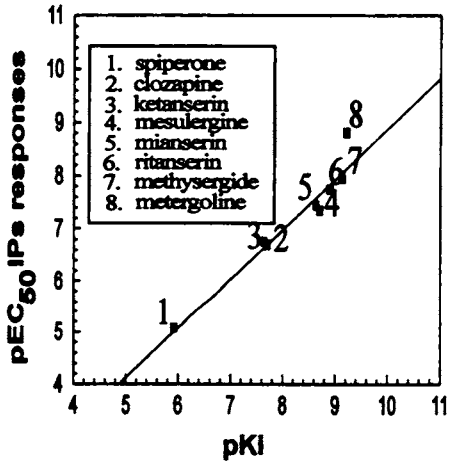

Fig. 4. Correlation of inverse agonist potencies and binding affinities for the baculovirus-expressed 5-HT$_{2C}$ receptor. A linear regression was performed between values obtained for binding affinity (pK_i) (Table 1) and for inhibition of spontaneous 5-HT$_{2C}$ receptor activity (pEC$_{50}$). A significant correlation (r^2 = 0.93, slope = 0.96, p < 0.0001) was obtained. Spiroxatrine was not included in the linear regression because it lacked inverse agonist activity in Sf9 cells.

suggest that the different antagonists show varying degrees of inverse efficacy, with spiroxatrine acting as a "neutral" antagonist at the 5-HT_{2C} receptor in this system.

In an effort to further analyze the inverse agonist effect and to assess the possibility that the inhibitory effect of antagonists was due to the presence of agonist (i.e., 5-HT) in the medium, additional experiments were performed using the apparently neutral antagonist spiroxatrine. As shown in Fig. 5A, spiroxatrine inhibited 5-HT-stimulated IP production in whole cells (analyzed at 20 hr after infection) in a manner expected for competition for occupancy of a common site. The IC_{50} for this effect (9 μM) was in good agreement with the affinity of spiroxatrine measured in binding studies (6.8 μM). Spiroxatrine also blocked the inhibitory effect of mianserin on basal IP production (Fig. 5B). Increasing concentrations of spiroxatrine produced a rightward shift in the dose-response curve for mianserin inhibition, consistent with the view that the two ligands bind to a common site. Analysis of the spiroxatrine data was carried out according to the method of Arunlakshana and Schild (24) (Fig. 5B, *inset*). The potency (pA_2) of spiroxatrine in reversing mianserin inhibition, as calculated from the Schild plot, was 5 μM, a value that agrees well with those obtained for spiroxatrine inhibition of mesulergine binding and inhibition of 5-HT-stimulated IP production (Table 1). Spiroxatrine thus appears to inhibit the binding of both 5-HT and mianserin, inhibiting their respective effects on IP production. These results are not consistent with the interpretation that the inhibition of basal activity by antagonists is due to the presence of agonist during the assay but, rather, support the view that the baculovirus-expressed 5-HT_{2C} receptor exhibits spontaneous activity that is inhibited by antagonists possessing inverse agonist activity.

Apparent down-regulation of 5-HT_{2C} receptors by antagonists. Serotonergic antagonists have been shown to promote the down-regulation of 5-HT_{2C} receptors in rat brain after chronic treatments *in vivo* (25–31). This effect of antagonists on 5-HT_{2C} receptor numbers has been reported to occur in primary cultures of choroid plexus but not in a transfected mammalian cell line expressing the cloned receptor (6). It was thus of interest to examine whether antagonist treatments could alter the level of baculovirus-expressed 5-HT_{2C} receptor binding sites.

Initial experiments were carried out by treatment of intact, 5-HT_{2C} receptor-expressing, Sf9 cells (at 38 hr after infection) for 12 hr with high concentrations (100 μM) of various drugs added to the culture medium. The drugs were then washed out as described in Materials and Methods, and the effect of pretreatment on receptor levels was determined by measuring the binding of [^{3}H]mesulergine (10 nM) using the standard filtration assay. As shown in Fig. 6, antagonist treatment resulted in decreases in [^{3}H]mesulergine binding, with the extent of the decrease varying from >90% for metergoline and ritanserin to little or no decrease for spiperone. The agonists 5-HT and DOI produced no significant change in the level of binding. When these experiments were carried out using membrane preparations, essentially the same profile of drug effects on the level of [^{3}H]mesulergine binding was observed as in whole cells (Fig. 6). For certain antago-

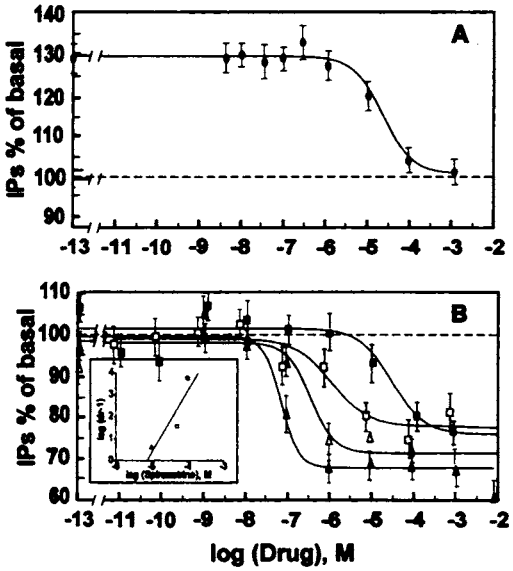

Fig. 5. Inhibition of inverse agonist activity by spiroxatrine. Sf9 cells labeled with *myo*-[^{3}H]inositol (1 μCi/ml) were infected for 20 hr (A) or 38 hr (B) with the 5-HT_{2C} receptor baculovirus. A, IP release measured with 1 μM 5-HT in the presence of spiroxatrine at the concentration indicated (*abscissa*). The IC_{50} for spiroxatrine inhibition of 5-HT-induced IP release was 9.12 ± 3 μM. B, IP release measured with the indicated concentrations of mianserim alone (▲) and in the presence of 10 μM (△), 50 μM (□), and 100 μM spiroxatrine (■), as described in Materials and Methods. Results were scaled by taking the level of IPs measured in the absence of added ligand as 100%, and the scaled data from three independent experiments were averaged to yield the points shown (*error bars*, standard error). Schild analysis (B, *inset*) was performed using EC_{50} values from each dose-response curve, i.e., 0.323 ± 0.100 μM (△), 1.540 ± 0.982 μM (□), and 16.500 ± 4.000 μM (■). The estimated pA_2 value for spiroxatrine was 5.49 (3.23 ± 2.5 μM) (r^2 = 0.90, slope = 1.63).

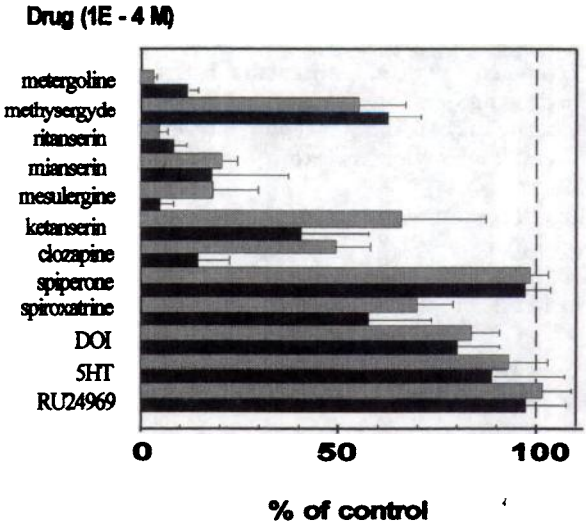

Fig. 6. Apparent loss of [^{3}H]mesulergine binding after antagonist pretreatment in whole cells and membranes. Sf9 cells infected for 38 hr with the 5-HT_{2C} receptor baculovirus were treated with the indicated ligand at 100 μM, up to 50 hr after infection (12-hr treatment). Cells were then washed extensively (see Materials and Methods), and [^{3}H]mesulergine binding was measured (■). In similar experiments, membranes from untreated 5-HT_{2C} receptor-expressing Sf9 cells (48 hr after infection) were treated with drugs (100 μM) for 1 hr in binding buffer (as described in Materials and Methods), followed by extensive washing, and [^{3}H]mesulergine (10 nM) binding was then measured (■). The data were scaled by taking the level of [^{3}H]mesulergine binding in the control samples as 100%, and the results shown represent the average ± standard error of the scaled values from three independent experiments.

nists, particularly mesulergine and clozapine, the magnitude of the decrease in binding sites was somewhat greater in membranes than in whole cells, but the rank orders for loss of sites were the same in cells and in membranes.

The loss of [^{3}H]mesulergine binding sites induced by antagonists was characterized in greater detail in membrane preparations. As shown in Fig. 7, pretreatment with varying concentrations of the different antagonists, followed by extensive washing to remove bound antagonist, produced dose-dependent decreases in [^{3}H]mesulergine binding. The potencies (see below) of the antagonists in reducing the binding of [^{3}H]mesulergine varied over several orders of magnitude, with metergoline being the most potent and spiperone having no effect at concentrations up to 1 mM. Treatment of membranes with 10 nM metergoline resulted in equivalent decreases (approximately 90%) in the binding of [^{3}H]mesulergine (10 nM) and [^{3}H]5-HT (350 nM) (data not shown), indicating that the binding of antagonists and that of agonists were equally affected by antagonist pretreatment.

To exclude the possibility that the loss of sites was simply due to residual antagonist remaining bound to the receptor after the wash, membranes were treated with either mianserin (1 μM), ritanserin (10 nM), or metergoline (10 nM) and washed, and saturation binding was performed with [^{3}H]mesulergine. As shown in Table 2, the antagonists produced marked decreases in B_{max} values but did not alter the apparent affinity of [^{3}H]mesulergine. Although ritanserin treatment seemed to slightly increase the K_d, the analysis of variance indicated that this change was not statistically significant ($\alpha = 0.001$, $p > 0.01$). This result suggests that the reduction in the number of [^{3}H]mesulergine binding sites for mianserin, metergoline, or ritanserin is not due to residual antagonist reversibly bound to the receptor.

The possibility of an artifact due to residual antagonist was also addressed by analyzing the association/dissociation of [^{3}H]mesulergine, an antagonist that itself causes a decrease in binding sites (Fig. 7). The time course of association of 20 nM [^{3}H]mesulergine, measured in membranes by filtration at different time points after radioligand addition, exhibited first-order kinetics, reaching a steady state within 60 min at 27° (Fig. 8). In parallel experiments, membranes were incu-

TABLE 2

Binding affinity of [^{3}H]mesulergine after apparent loss of 5-HT$_{2C}$ receptor sites expressed in Sf9 cells

Mesulergine binding affinity (K_d) and receptor density (B_{max}) were measured in membranes (from cells 48 hr after infection) that had been preincubated for 60 min with drugs and then extensively washed before the saturation binding analysis, as described in Materials and Methods. Results from antagonist treatment of membranes were determined from three independent experiments, with mean values and standard errors calculated from separate estimates fitted with the program LIGAND.

Treatments	[^{3}H]Mesulergine binding parameters		
	K_d[a]	B_{max}[b]	
	nM	pmol/mg	% of control
Control	2.1 ± 1.2	22.0 ± 2.0	100 ± 9
Mianserin (1 μM)	3.8 ± 2.2	14.5 ± 2.0	66 ± 9
Metergoline (10 nM)	2.0 ± 0.6	2.9 ± 1.1	12 ± 6
Ritanserin (10 nM)	7.6 ± 3.0	5.5 ± 3.0	25 ± 12

[a] Analysis of variance was performed K_d estimates for control and drug-treated membranes ($p > 0.01$).

[b] Analysis of variance was performed on B_{max} estimates with the rejected null hypothesis ($p < 0.001$). One-tailed Dunnett's test (H_o, $\mu_{control} > \mu_{drug}$, $\alpha = 0.05$) was used to compare drug treatment B_{max} values with control.

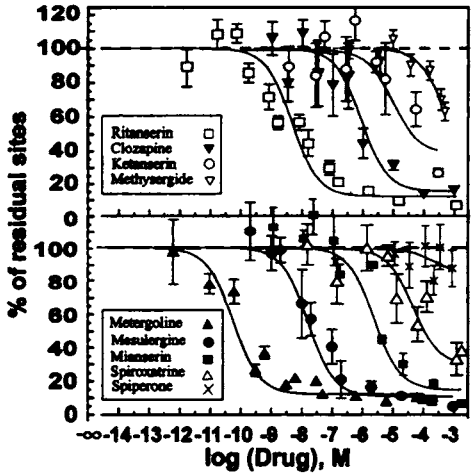

Fig. 7. Effects of serotonergic antagonists on the apparent loss of [^{3}H]mesulergine binding sites in 5-HT$_{2C}$ receptor-containing Sf9 membranes after antagonist pretreatment. Membranes prepared (48 hr after infection) from Sf9 cells infected with the 5-HT$_{2C}$ receptor baculovirus were treated with the indicated 5-HT$_{2C}$ receptor antagonists for 1 hr. Treated and control membranes were washed extensively in parallel (see Materials and Methods) and then incubated with [^{3}H]mesulergine (10 nM) to determine the residual binding after drug treatment. For each ligand, data from three independent experiments were analyzed simultaneously, assuming a common value for EC_{50} (Table 3), with $Y_{(X\to 0)}$ and $Y_{(X\to\infty)}$ being unconstrained. The curves and averaged points (three determinations) shown were derived from a representative experiment, with all drugs being tested in parallel with the same membranes on the same day. Values from a representative set of data were scaled by taking the fitted values of $Y_{(X\to 0)}$ as 100%, and the *points* shown represent the averages ± standard errors of the scaled data. Each *curve* was generated using the fitted values of EC_{50}, with $Y_{(X\to 0)}$ being set to 100% and $Y_{(X\to\infty)}$ being set to the average of $[[Y_{(X\to\infty)}/Y_{(X\to 0)}] \cdot 100\%]$. For clarity, points corresponding to concentrations less than EC_{50}/1000 were omitted from the figure. EC_{50} values are reported in Table 3.

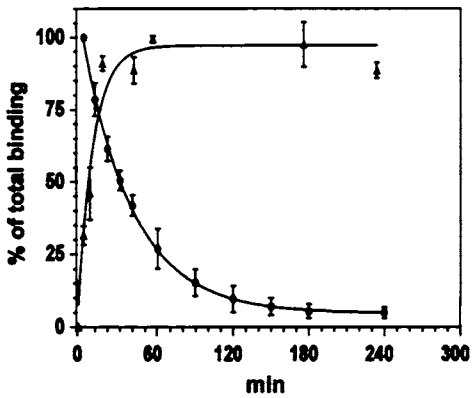

Fig. 8. Time dependence of [^{3}H]mesulergine binding in membranes. Binding of [^{3}H]mesulergine to membranes from Sf9 cells expressing the 5-HT$_{2C}$ receptor (B_{max} = 27 pmol/mg of membrane protein) was measured at the times indicated (▲). Dissociation of labeled mesulergine (after a 2-hr incubation with 500 nM [^{3}H]mesulergine isotopically diluted to 8 Ci/mmol) was measured at different time points after a quick centrifugation and 10,000-fold dilution in binding buffer, as described in Materials and Methods (●). Data from three independent experiments were averaged and expressed as a percentage of total binding (*error bars*, standard error). The averaged data were fitted adequately by assuming one-phase exponential decay (●) or association (▲). Pseudo-first-order kinetics were observed at 27° for both [^{3}H]mesulergine association (20 nM, $t_{1/2}$ = 10 min) and dissociation (500 nM, $t_{1/2}$ = 40 min). Control dilution of untreated membranes, performed in parallel, did not affect their ligand binding capacity (data not shown).

bated for 60 min with [^{3}H]mesulergine at 500 nM, a concentration sufficient to cause a >50% reduction in binding sites (Fig. 7). In the latter experiments, dissociation of the bound [^{3}H]mesulergine was then initiated by centrifuging the membranes and resuspending them in a large volume of buffer (1×10^5 times the original incubation volume), and residual bound ligand was measured at various times using a filtration assay (see the legend to Fig. 8). As shown in Fig. 8, approximately 95% of the bound [^{3}H]mesulergine dissociated from the membranes in a 2-hr incubation at 27° ($t_{1/2}$ = 40 min). When these membranes were then tested for their ability to bind 10 nM [^{3}H]mesulergine, the total binding was reduced by approximately 60%, compared with control membranes (i.e., no mesulergine pretreatment) processed in parallel, consistent with the effect of 500 nM unlabeled mesulergine (Fig. 7). These results clearly demonstrate that the ability of mesulergine (and presumably the other antagonists) to reduce the number of binding sites is not due to residual ligand occupying the receptor, because the lost binding sites were not recovered even after dissociation of >95% of the bound mesulergine.

To determine whether the loss of sites produced by antagonist treatment was a reversible process, intact cells or membranes were treated with metergoline (10 nM), washed according to the standard protocol, and incubated for up to 24 hr in the absence of added drug. Analysis of [^{3}H]mesulergine binding after such treatments showed no recovery of binding sites, whereas control samples (i.e., no metergoline pretreatment) processed in parallel exhibited only a slight decrease in [^{3}H]mesulergine binding (data not shown). The loss of sites for the baculovirus-expressed 5-HT$_{2C}$ receptor after antagonist treatment thus appears to be irreversible under the conditions examined here.

Lack of correlation between inverse agonism and loss of binding sites. The dose-response curves for loss of binding sites after agonist pretreatment (Fig. 7) were analyzed (Table 2) to generate EC$_{50}$ (i.e., concentration producing a 50% decrease in binding) values for the series of antagonists. The results (Table 3) provided the following rank order of potency for loss of sites: metergoline > ritanserin ≥ mesulergine > clozapine > mianserin > spiroxatrine ≥ ketanserin ≫ methysergide. This clearly differs from the rank orders determined for binding affinity and inverse agonist activity (Table 1). Comparison of the EC$_{50}$ values for inverse agonism versus loss of binding sites (Table 3) indicated that certain antagonists (metergoline and spiroxatrine) were highly selective in decreasing binding sites, whereas others (methysergide, ketanserin, and spiperone) were selective in inhibiting spontaneous receptor activity. Although the antagonists showed a wide range of potencies in reducing the level of binding sites, the results in Fig. 7 suggest that the maximal reduction was the same (approximately 90% loss of sites) for all of the antagonists, although the effects of spiroxatrine and methysergide appeared to be incomplete at the highest concentrations used. This contrasts with the results for inverse agonism (Fig. 3; Table 1), which indicated that the maximal inhibition of spontaneous receptor activity varied depending on the antagonist. The present findings thus strongly suggest that the inverse agonist activity and reduction of binding sites represent distinct actions of serotonergic antagonists on the 5-HT$_{2C}$ receptor.

TABLE 3

Apparent loss of binding sites induced by antagonists with 5-HT$_{2C}$ receptors expressed in Sf9 cells

Residual binding sites were estimated in membranes (from 48-hr infected cells) that had been pretreated for 60 min with drugs at different concentrations and then extensively washed before the binding analysis, as described in Materials and Methods. Nonspecific binding was determined with 10 μM mianserin. Antagonist potencies (EC$_{50}$) (mean ± standard error) for inducing an apparent loss of sites were taken from Fig. 7.

Antagonists	Apparent loss of [^{3}H]mesulergine binding sites		
	$-$pEC$_{50}$	90 Decrease at 10 × K_i[a]	Selectivity ratio[b]
Metergoline	10.25 ± 0.12	79 ± 7	0.036
Methysergide	>3	0	>1 × 10^6
Ritanserin	8.42 ± 0.16	49 ± 15	0.32
Mianserin	5.60 ± 0.15	0	72.6
Mesulergine	7.98 ± 0.09	38 ± 7	0.23
Ketanserin	4.45 ± 0.07	2 ± 5	1.76
Clozapine	6.05 ± 0.16	10 ± 7	5.1
Spiperone	ND[c]	0	>1 × 10^6
Spiroxatrine	4.64 ± 0.03	35 ± 5	<1 × 10^{-5}

[a] The loss of [^{3}H]mesulergine binding with antagonist occupancy at 10 × K_i was extrapolated from Fig. 7.

[b] Selectivity ratio = (EC$_{50}$ for loss of binding/EC$_{50}$ for inverse agonism) (Table 1). Values of <1, selective for loss of binding; values of >1, selective for inverse agonism.

[c] ND, not determined.

Discussion

The antagonist pharmacology of the 5-HT$_{2C}$ receptor is an area of considerable interest, due to its potential therapeutic relevance to the action of antipsychotic drugs. Studies using a variety of model systems, including intact animals, primary cultures, and mammalian expression systems, have provided evidence that serotonergic antagonists exert multiple actions on the 5-HT$_{2C}$ receptor. In addition to inhibiting receptor activation by 5-HT, antagonists have been reported to inhibit spontaneous activity of the recombinant receptor in 3T3 cells (6, 7) and to promote down-regulation of the 5-HT$_{2C}$ receptor in rat brain *in vivo* (25–31) and in choroid plexus *in vitro* (6). The present study provides the first detailed comparison of these different modes of antagonist action in a single system based on the expression of the cloned 5-HT$_{2C}$ receptor in the baculovirus/insect cell system.

The results of this study show that the infection of Sf9 cells with recombinant baculovirus encoding the 5-HT$_{2C}$ receptor leads to the expression of functional receptors capable of regulating intracellular levels of IPs. The levels of 5-HT$_{2C}$ receptor expression observed in the Sf9/baculovirus system (approximately 1×10^6 sites/cell in intact cells and 27 pmol/mg of protein in membrane preparations) are roughly 20-fold higher than those of the native receptor in rat choroid plexus (26, 28) and >5-fold higher than the level reported for NIH/3T3 cells expressing the recombinant receptor (6). As observed with mammalian systems expressing either native or recombinant 5-HT$_{2C}$ receptors, the baculovirus-expressed 5-HT$_{2C}$ receptor activates polyphosphoinositide hydrolysis in response to serotonergic agonists, via a pertussis toxin-insensitive pathway. This is consistent with results of previous studies (32, 33), which indicated that Sf9 cells possess a G$_q$-like G protein, an endogenous phospholipase C, and Ca^{2+} signaling mechanisms that are amenable to activation by mammalian G protein-coupled receptors. The potencies of 5-HT and DOI in stimulating IP production in Sf9 cells expressing the 5-HT$_{2C}$ receptor (19 nM and 100 nM, respec-

tively) were comparable to those reported for choroid plexus polyphosphoinositide hydrolysis (30, 31). The maximal level of stimulation of IP production in these experiments (30% over basal levels at 20 hr after infection), however, was considerably lower than that observed in either choroid plexus (approximately 400% stimulation) (30, 31) or NIH/3T3 cells (approximately 200% stimulation) (6). The small response to agonists of the baculovirus-expressed receptor in this study could conceivably reflect partial incompatibility between the mammalian receptor and the insect G protein-effector relay. Alternatively, poor responsiveness to agonists could be a consequence of the high levels of receptors produced in this system. Agonist-independent effector activation by 5-HT_{2C} (Fig. 1) and β_2-adrenergic (8) receptors in Sf9 cells increases with the level of receptor expression, and it follows that inhibitory feedback mechanisms may alter the activity of intracellular components involved in second messenger production and/or metabolism.

Agonist-independent increases in IPs in Sf9 cells expressing the 5-HT_{2C} receptor (Fig. 1) indicate that the receptor is spontaneously active when expressed in this system. Spontaneous activity appeared to reach a maximum approximately 38 hr after infection and was inhibited by a variety of 5-HT_{2C} receptor antagonists. These observations confirm similar findings with the 5-HT_{2C} receptor expressed in NIH/3T3 cells (6, 7). An excellent correlation was observed in the present study between the binding affinities of these antagonists and their potencies (EC_{50} values) as inverse agonists. The results further show that 5-HT_{2C} receptor inverse agonists display a range of intrinsic activities in Sf9 cells (Table 1), as observed for the β_2-adrenergic receptor expressed in Sf9 and mammalian cells (8), as well as for the δ-opioid receptor (13) and the α_2-adrenergic receptor (11). These findings are consistent with the idea that intrinsic activity can vary among inverse agonists at a particular G protein-coupled receptor, similarly to the range of activities typically exhibited by full and partial agonists. In contrast, results reported for the 5-HT_{2C} receptor in NIH/3T3 cells (6, 7) suggested that antagonists either fully inhibit or are without effect on spontaneous receptor activity in that system. In addition, methysergide was reported to behave as a neutral antagonist at the 5-HT_{2C} receptor in the mammalian cell line but was clearly an efficacious inverse agonist in the Sf9 cells. Other ligands assayed in both Sf9 and NIH/3T3 cells (ketanserin, spiperone, mesulergine, mianserin, and metergoline) inhibited spontaneous 5-HT_{2C} receptor activity in both systems. The reason for the apparent discrepancy in methysergide activity in the two cell lines is unclear.

There is increasing evidence that inverse agonism is a general phenomenon at G protein-coupled receptors. In addition to the 5-HT_{2C} receptor, the inhibition of spontaneous activity has been observed with recombinant β_2-adrenergic (8), α_2-adrenergic (11), and D_1 dopaminergic (9) receptors expressed from cloned cDNAs in cultured cell lines, the δ-opioid receptor in NG108 cells (13), and the M_2 muscarinic (12) and B_2 bradykinin (10) receptors in tissue-derived receptor preparations. In these reports, most of the antagonists studied appeared to negatively regulate the activity of the receptor, with relatively few being devoid of intrinsic activity (i.e., neutral ligands). The results of these studies support the general view that G protein-coupled receptors can assume inactive and active states, with the net effect of a given ligand being determined by its relative binding preference for the available states of the receptor.

A major finding of the present study is the demonstration that serotonergic antagonist treatment of intact Sf9 cells or membranes containing the baculovirus-expressed 5-HT_{2C} receptor, followed by removal of the drug by extensive washing, resulted in a loss of binding capacity for both the antagonist [^{3}H]mesulergine and the agonist [^{3}H]5-HT. Several observations strongly suggest that this action of antagonists in Sf9 cells is related to the ability of these drugs to decrease the level of 5-HT_{2C} receptors in various brain regions after chronic administration *in vivo* (25–31). There was a striking correspondence between the relative abilities of antagonists to promote such atypical down-regulation of 5-HT_{2C} receptors *in vivo* and the potencies of the same antagonists in decreasing [^{3}H]mesulergine binding to the baculovirus-expressed 5-HT_{2C} receptor. Although quantitative comparisons are complicated by the nature of *in vivo* experiments (variations in treatment regimens in different studies and the lack of knowledge on drug concentrations in tissues), the ligands exhibiting the highest potencies in reducing sites in Sf9 cell membranes (metergoline, ritanserin, and mesulergine) were the same ones that produced the greatest decrease in 5-HT_{2C} receptor levels after *in vivo* treatments (25). Similarly, antagonists such as mianserin, ketaserin, and clozapine, which produced a lesser but demonstrable effect on receptor levels *in vivo* (25–28), showed intermediate potencies at the baculovirus-expressed receptor, whereas spiperone seemed to be without effect on receptor levels in either system (25).

Another common feature of the antagonist effect on 5-HT_{2C} receptor levels *in vivo* and in Sf9 cells was the apparent dose dependence of this action. This was clearly evident for the baculovirus-expressed receptor in membranes in the present study and has been reported for the effect of clozapine on 5-HT_{2C} receptor numbers in choroid plexus after chronic treatment of rats (26). Finally, results from *in vivo* studies indicate that the reduction in 5-HT_{2C} receptor number by antagonist treatment is irreversible, with the recovery of sites after cessation of treatment requiring synthesis of new receptors. Experiments performed with metergoline in the present study failed to detect recovery of lost binding sites in membranes or intact cells up to 24 hr after removal of the antagonist, consistent with an irreversible loss of receptor binding capacity.

One obvious difference in the action of antagonists in reducing the numbers of 5-HT_{2C} receptors *in vivo* and in Sf9 cells was the time course of this effect. For the most potent antagonists, the maximal effect in membranes from Sf9 cells (approximately 90% loss of sites) was reached within 1 hr of treatment; similar decreases were observed after 24-hr treatment of intact Sf9 cells (the only time point examined in this study). In contrast, the same drugs generally required several days of repeated administration to produce comparable effects in brain regions (25, 26, 28, 29) or in primary cultures of choroid plexus (6, 28). One possible interpretation of this difference is that the ability of antagonists to promote loss of binding capacity of the 5-HT_{2C} receptor depends on the cellular context. The observation that NIH/3T3 cells expressing the cloned 5-HT_{2C} receptor do not show reduced receptor numbers after prolonged antagonist treatment (6) tends to support this view. The rapid time course for antagonist-

promoted loss of 5-HT_{2C} receptors in Sf9 cells observed in the present study and the fact that this process could be reproduced in membrane preparations indicate that the baculovirus/Sf9 insect cell system is particularly permissive for this action of antagonists. Whether this is due to the high levels of receptor expression in this system, the presence or absence of other proteins regulating this process, or physico-chemical properties of the insect cell membrane bilayer remains to be determined. The striking parallels observed between the actions of antagonists in reducing 5-HT_{2C} receptor numbers *in vivo* and in Sf9 cells, however, strongly suggest that the two phenomena reflect the same underlying process.

The biochemical mechanisms leading to the reduction in receptor numbers after antagonist treatments are obscure. Studies of the *in vivo* effect, including quantification of residual antagonist in brain tissue (26) and measurement of radioligand affinity after down-regulation (25, 26, 28, 29), suggested that the reduction in binding sites was not due to occupation of receptors by residual drug. This possible artifact clearly can be ruled out for the effect of antagonists in Sf9 cell membranes, because 1) the apparent dissociation constant for [^{3}H]mesulergine was not increased after treatments with three different antagonists that produced significant reductions in the number of binding sites (up to 90% for metergoline) and 2) the loss of binding sites produced by treatment with isotopically diluted [^{3}H]mesulergine persisted after removal of 95% of the bound [^{3}H]mesulergine, as quantified in dissociation experiments. The results thus support the view that the loss of binding sites reflects a decrease in receptor number in response to antagonist treatment, rather than continued occupation of the receptors by drug. The present findings are also consistent with previous studies using primary cultures of choroid plexus epithelial cells, which demonstrated that antagonists exert this effect through a direct action on receptor-expressing cells and that loss of binding sites does not involve changes in receptor mRNA levels (6).

It was recently proposed (6) that the abilities of antagonists to decrease 5-HT_{2C} receptor number were directly correlated with their inverse agonist activities. This suggestion was based on studies showing that mianserin, an inverse agonist at the 5-HT_{2C} receptor in NIH/3T3 cells, promoted loss of binding sites in cultured choroid plexus cells, whereas bromo-*d*-lysergic acid diethylamide, a neutral antagonist in NIH/3T3 cells, had no effect on receptor numbers in choroid plexus. Unfortunately, that study did not permit direct comparison of antagonist actions on the 5-HT_{2C} receptor in the same cellular system, because choroid plexus did not exhibit antagonist-inhibited spontaneous receptor activity and NIH/3T3 cells did not show antagonist-induced down-regulation of the 5-HT_{2C} receptor. The inability to detect the two effects of antagonists in the same model system could indicate that they occur through distinct mechanisms.

The results of the present study, in which antagonist potencies for inhibition of spontaneous receptor activity and loss of binding capacity were directly compared for the baculovirus-expressed 5-HT_{2C} receptor, suggest that the two effects may reflect distinct actions of antagonists. Although most of the antagonists tested were able to elicit both types of responses, the rank orders of potency were clearly different for the two effects, with certain antagonists showing a high degree of selectivity for one or the other action (Table 3). Furthermore, whereas inverse agonist activity was directly correlated with receptor occupancy (Fig. 4), this did not appear to be the case for the decrease in receptor numbers produced by antagonist treatments. As shown in Table 3, treatment of 5-HT_{2C} receptor-containing membranes with antagonists at concentrations 10 times their respective binding affinities (sufficient to occupy >90% of the available sites at equilibrium) produced decreases in [^{3}H]mesulergine binding ranging from no effect (methysergide, mianserin, and spiperone) to the loss of approximately 85% of binding sites (metergoline). The results suggest that the antagonists differ with respect to their propensities to reduce receptor number.

The actions of serotonergic antagonists in reducing the numbers of 5-HT_{2C} receptors show similarities to the better characterized phenomenon of agonist-induced receptor down-regulation. In the case of the β_2-adrenergic receptor, continued exposure to high concentrations of agonist results in a reduction in the number of receptors through a process believed to involve internalization of agonist-receptor complexes and subsequent proteolysis in lysosomes (see Ref. 34 for review). Although it is difficult to conceive that such a process could be occurring in Sf9 cell membrane preparations containing the 5-HT_{2C} receptor, a recent study (35) has provided evidence that internalization of the β_2-adrenergic receptor may involve an initial agonist-induced rearrangement of receptors at the cell surface. Whether the action of antagonists on the 5-HT_{2C} receptor observed in the present study reflects such a preinternalization event is an intriguing question for further work.

The present findings emphasize important questions regarding the mechanism of action of antagonists as therapeutic agents. According to the "classical" view, antagonists act by blocking the stimulatory effect of the endogenous transmitter but have no direct actions themselves. The growing literature on inverse agonism at G protein-coupled receptors raises the possibility that antagonists may exert effects through inhibition of spontaneous receptor activity. The relative contribution of this mode of action *in vivo* will obviously depend on the occurrence of spontaneously active receptors. Although such spontaneous activity has been detected in tissue preparations (10, 12) and is likely to be of significance in the case of receptor mutants showing constitutive activity (36), additional studies are required to establish the potential therapeutic relevance of inverse agonism. The ability of antagonists to promote a reduction in receptor number, as characterized in the present study for the 5-HT_{2C} receptor, would appear to represent a third mechanism of antagonist action. Atypical down-regulation by antagonists has been described for both the 5-HT_{2C} and 5-HT_{2A} receptors, and numerous authors have proposed that this effect of antagonists may underlie the therapeutic effects of certain antipsychotic and antidepressant drugs (see Refs. 4 and 5 for review). The list of antagonists found to promote loss of binding to the baculovirus-expressed 5-HT_{2C} receptor in the present study includes drugs in current therapeutic use, namely the antipsychotic agent clozapine and the antidepressant mianserin. It will be interesting to determine whether other G protein-coupled receptors, such as the 5-HT_{2A} receptor, show similar antagonist profiles when expressed in insect cells. It is tempting to speculate that the activity profiles of receptor antagonists with baculovirus-expressed receptors, particularly the selectivity for inverse agonism versus receptor

down-regulation, could provide a useful index for ranking the therapeutic potential of new drug candidates.

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