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Effects of Agonists, Partial Agonists, and Antagonists on the Regulation of 5-Hydroxytryptamine$_2$ Receptors in P11 Cells

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SUMMARY

Studies of the regulation of 5-hydroxytryptamine (5-HT$_2$) receptors *in vivo* have generated anomalous and sometimes contradictory results. In particular, administration of antagonists unexpectedly results in a reduction in the density of 5-HT$_2$ receptors. P11 cells, which express a high density of 5-HT$_2$ receptors coupled to phosphoinositide hydrolysis, were used to investigate the regulation of receptors *in vitro* by agonists, partial agonists, and antagonists. (±)-2,5-Dimethoxy-4-iodophenylisopropylamine (DOI) and (+)-lysergic acid diethylamide (LSD) caused marked reductions in the density of 5-HT$_2$ receptors as has been observed *in vivo*. Down-regulation was prevented by coincubation with ketanserin. The decrease in the density of 5-HT$_2$ receptors after exposure to 5-HT, LSD, or DOI was time dependent and was not a consequence of residual drug in binding assays or irreversibly bound drug. The ability of 5-HT, DOI, and LSD to down-regulate 5-HT$_2$ receptors was not proportional to the ability of these compounds to stimulate phosphoinositide hydrolysis. Ketanserin and mianserin, antagonists which cause paradoxical decreases in the density of 5-HT$_2$ receptors *in vivo*, did not alter the density of 5-HT$_2$ receptors on P11 cells, even after prolonged incubation with drug. Results of the current studies, which demonstrate agonist- but not antagonist-induced down-regulation of 5-HT$_2$ receptors, lead to the conclusion that the ability of ketanserin and mianserin to down-regulate receptors *in vivo* is the result of indirect actions of these drugs and is unlikely to be a direct consequence of receptor occupancy by antagonists.

Many actions of 5-HT in the periphery and in the central nervous system are mediated by an interaction with 5-HT$_2$ receptors. Changes in the density of 5-HT$_2$ receptors in the central nervous system have been documented in studies of postmortem tissue from patients diagnosed with Parkinson's disease (1, 2), Alzheimer's disease (1, 2), and chronic schizophrenia (3). Additionally, blockade of 5-HT$_2$ receptor function with antagonists has proven beneficial in the clinical management of anxiety disorders, major depression, and schizophrenia (4–6). These and other findings have prompted numerous investigations of the regulation of 5-HT$_2$ receptors. The results of these investigations have revealed an unexpected pattern of regulation of 5-HT$_2$ receptors *in vivo* that is only partially consistent with regulatory responses that have been observed for other members of the GTP-binding protein-linked receptor family (7, 8).

The effects of administration of 5-HT$_2$ receptor agonists on the density of 5-HT$_2$ receptors have been described. Blackshear *et al.* (9) reported that treatment of rats with trifluoromethylphenylpiperazine for 5 days leads to a decrease in the density of cortical [^{3}H]ketanserin binding sites. Similar results have been obtained after administration of quipazine, a selective 5-HT$_2$ receptor agonist (10). The daily administration of LSD (130 μg/kg) for 5 days results in a selective decrease in [^{3}H]ketanserin binding to 5-HT$_2$ receptors in cortex, hippocampus, and midbrain (11). This decrease in the density of 5-HT$_2$ receptors was observed to parallel the development of behavioral tolerance seen after repeated administration of LSD. In a later study, Buckholtz *et al.* (12) reported that a single high dose (650 μg/kg) of LSD decreases the number of 5-HT$_2$ receptors within 4 hr after drug administration.

More recently, hallucinogenic derivatives of (±)-2,5-dimethoxyphenylisopropylamine, including DOI and DOM, have been shown to be 5-HT$_2$ agonists (13, 14). Behavioral (15–17) and neurophysiological (18, 19) effects of these compounds are blocked by 5-HT$_2$ receptor antagonists, suggesting that stimulation of 5-HT$_2$ receptors is involved in mediating their effects. Chronic administration of DOI (1.0 mg/kg) results in a decrease in 5-HT$_2$ receptors, as measured by binding of [^{3}H]ketanserin (12) or ^{125}I-LSD (20). Single injections of DOI also reduce the density of receptors labeled with [^{3}H]DOB or [^{3}H]ketanserin (21). Taken together, these studies demonstrate that 5-HT$_2$ receptors are down-regulated by agonists, a finding that is

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); B_{max}, maximal density of receptors; DOI, (±)-2,5-dimethoxy-4-iodophenylisopropylamine; DOM, (±)-2,5-dimethoxy-4-methylphenylisopropylamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LSD, (+)-lysergic acid diethylamide; PI, phosphoinositide.

similar to the results of studies on many other neurotransmitter receptors (7, 8).

Unlike responses observed after administration of agonists, other aspects of the regulation of 5-HT$_2$ receptors *in vivo* are less easily explained. For example, 5-HT$_2$ receptors are not up-regulated after lesion of serotonergic neurons with 5,7-dihydroxytryptamine (10, 22) or *para*-chloroamphetamine (23). In contrast, depletion of catecholamines from noradrenergic and dopaminergic nerve terminals by 6-hydroxydopamine leads to marked increases in the density of catecholamine receptors (24, 25). In addition, it has been reported that chronic administration of selective inhibitors of serotonin uptake (e.g., fluoxetine) does not cause down-regulation of 5-HT$_2$ receptors (26). These unexpected findings may be explained by low endogenous serotonergic tone (27, 28) or noninnervated receptors (27), although direct evidence to support these hypotheses has not been obtained.

More surprising are the changes in the density of 5-HT$_2$ receptors observed after repeated administration of 5-HT$_2$ receptor antagonists to rats. Unexpectedly, chronic blockade of 5-HT$_2$ receptors by antagonists, including metergoline (29), ketanserin (21, 30), ritanserin (31), and setoperone (31, 32), leads to a decrease in the density of 5-HT$_2$ receptors. Paradoxical decreases in the density of 5-HT$_2$ receptors have also been observed after single and repeated injections of the atypical antidepressant mianserin (33). Antagonist-induced down-regulation of 5-HT$_2$ receptors is unexpected because chronic receptor blockade *in vivo* usually results in receptor up-regulation (see Ref. 8 for review). It has been suggested that antagonists of 5-HT$_2$ receptors may be weak partial agonists and that this property of the compounds could account for the observed effects. Stimulation of PI hydrolysis by these compounds has not been observed, however (34). Questions of residual drug in binding assays, the maintenance of complete receptor blockade during the course of administration of the drugs *in vivo*, and possible indirect effects of the compounds have complicated the interpretation of results of studies with antagonists. Thus, it has not been possible to determine whether the regulation of 5-HT$_2$ receptors after administration of antagonists is due to a unique regulatory mechanism or is the result of complexities inherent in the use of *in vivo* models.

Clonal cell lines are valuable alternative systems for studying the effects of drugs on the density of receptors and their coupling to second messenger systems. Until recently, few well characterized *in vitro* model systems have been available to study 5-HT$_2$ receptors. Smooth muscle cells from calf aorta (35) and cerebellar granule cells in culture (36) have been used to investigate second messenger responses coupled to 5-HT$_2$ receptor stimulation, but they do not express a sufficiently high density of receptors to permit radioligand binding studies.

P11 cells express a high density of 5-HT$_2$ receptors coupled to PI turnover and have been used previously as a model system to study effects of agonists on 5-HT$_2$ receptors (37, 38). Using P11 cells the effects of exposure to agonists (5-HT), partial agonists (LSD and DOI), and antagonists (ketanserin and mianserin) were explored to identify responses of 5-HT$_2$ receptors after direct interactions with these compounds. Similar to an earlier study of β-adrenergic receptors (39), agonist activity of the drugs used in this study was assessed by measuring ternary complex formation (as evidenced by GTP shifts in drug competition studies), second messenger production, and the ability to alter the density of 5-HT$_2$ receptors on the cells. Although GTP had no detectable effect on the inhibition by LSD and DOI of ^{125}I-LSD binding, both LSD and DOI were able to stimulate PI turnover and they caused marked decreases in the density of 5-HT$_2$ receptors, similar to the results of studies conducted *in vivo*. In contrast, the antagonists ketanserin and mianserin did not stimulate PI hydrolysis and did not alter the density of 5-HT$_2$ receptors in P11 cells. Based on these results, it appears that agonists and antagonists of 5-HT$_2$ receptors regulate receptor density *in vivo* by distinct mechanisms. It is hypothesized that the ability of antagonists to down-regulate 5-HT$_2$ receptors *in vivo* is the result of indirect actions of these drugs and is unlikely to be the result of direct occupation of 5-HT$_2$ receptors.

Experimental Procedures

Materials. *myo*-[^{3}H]Inositol (20 Ci/mmol) was purchased from New England Nuclear (Boston, MA). ^{125}I-LSD (2200 Ci/mmol) was purchased from New England Nuclear or was provided by Dr. H. Kung and Dr. Mei-Ping Kung at the University of Pennsylvania. (±)-DOI hydrochloride, ketanserin tartrate, and mianserin hydrochloride were purchased from Research Biochemicals Inc. (Natick, MA). Components for cell culture media were from GIBCO (Grand Island, NY). Other drugs and chemicals were from Sigma (St. Louis, MO).

Cell culture. P11 cells are a clonal cell line derived from rat pituitary tumor 7315a (37). Cells were grown in monolayer culture in high-glucose Dulbecco's modified Eagle medium (supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 150 μg/ml oxaloacetate, 50 μg/ml pyruvate, 0.2 units/ml insulin, 100 units/ml nystatin, and 10% charcoal-treated fetal bovine serum) in a humidified atmosphere containing 10% CO_2, at 37°. Cells were detached with a solution of 0.05% trypsin and 0.53 mM EDTA and were plated on uncoated 150-mm tissue culture plates at a density of 12,000–14,000 cells/cm^2. Cells were fed every third day. Treatment and harvesting of cells were initiated on days 4–6, when cells approached confluence.

Membrane preparation. Membranes from P11 cells treated with agonists and partial agonists were prepared and frozen as described previously (37). When assays were to be performed, frozen samples were thawed, homogenized in 30 ml of 50 mM Tris, and incubated at 37° for 30 min in the presence of 300 μM GTP. Samples were then centrifuged at 25,000 × *g* for 15 min, resuspended in 50 mM Tris, washed by centrifugation as described above, and resuspended in 50 mM Tris containing 300 μM GTP and 1 mM $MgCl_2$, for use in binding assays.

For experiments with antagonists, membranes were subjected to a more vigorous washing protocol to ensure complete removal of residual drug. Briefly, cells were washed with phosphate-buffered saline (pH 7.4), lysed in hypotonic buffer (5 mM HEPES, 5 mM EDTA, pH 7.5) for 10 min at 4°, removed from plates by scraping, and centrifuged at 25,000 × *g* for 15 min. The resulting pellets were resuspended in 25 ml of buffer containing 10 mM HEPES and 10 mM EDTA. Membranes were recovered by centrifugation, resuspended in 30 ml of buffer containing 20 mM HEPES, 140 mM NaCl, and 300 μM GTP, and incubated at 37° for 30 min. Samples were then centrifuged, and membranes were resuspended in 30 ml of 50 mM Tris (pH 7.4) containing 100 mM NaCl and were reincubated at 37° for 30 min. Samples were again centrifuged, and membranes were resuspended in 30 ml of 50 mM Tris, centrifuged, homogenized in a small volume of Tris buffer, frozen on dry ice, and stored at −80°. Before being assayed, samples were homogenized in 30 ml of 50 mM Tris and incubated at 37° for 30 min. Membranes were then centrifuged, resuspended in 50 mM Tris, recovered by centrifugation, and resuspended in 50 mM Tris containing 300 μM GTP and 1 mM $MgCl_2$.

Radioligand binding. ^{125}I-LSD (2200 Ci/mmol) was used to mea-

sure 5-HT_2 receptors on P11 cells. For saturation analysis, radioligand binding was initiated by the addition of 50 μl of tissue (20–80 μg of protein) to 25 μl of 50 mM Tris (pH 7.4) containing 0.1–2.0 nM ^{125}I-LSD. Specific binding was defined as binding inhibited by 1 μM ketanserin. The final assay volume was 100 μl. Assay tubes were allowed to incubate for 60 min at 37°. Reactions were terminated by the addition of 8 ml of ice-cold buffer (50 mM Tris, 100 mM NaCl), and membranes were collected on glass fiber filters, which had been soaked in 0.3% polyethylenimine, using a Millipore filter manifold or a Brandel cell harvester. Filters were washed twice with the same buffer, and radioactivity remaining on the filters was determined in a γ counter. Saturation binding data were transformed by the method of Scatchard (40). Protein was determined by the method of Lowry *et al.* (41).

In some experiments (Table 2 and Fig. 5), binding was carried out at two concentrations of radioligand. P11 cell membranes (20–40 μg of protein) were incubated with 0.2 nM or 1.0 nM ^{125}I-LSD for 60 min at 37°. Specific binding was determined for each concentration of radioligand. Results were calculated as a percentage of the specific binding detected in membranes from control cells incubated with the same concentrations of radioligand and assayed simultaneously. A mean of the percentage of control values for both concentrations of ligand was then calculated for each sample. For individual samples, there was good agreement between the two values. This served as an internal control in each experiment, confirming that the washing procedure had effectively removed exogenous drugs from each sample.

Measurement of PI hydrolysis. P11 cells were plated in six-well cluster dishes (Costar 3506) at a density of 15,000 cells/cm^2. After 24 hr the medium was replaced with medium containing *myo*-[^{3}H]inositol (3.5 μCi/well) and 3 days later, when cells were almost confluent, the medium was removed and replaced with Dulbecco's modified Eagle medium. LiCl was added to a final concentration of 20 mM, and 10 min later drugs were added. After a 40-min incubation, inositol phosphate accumulation was stopped by the addition of perchloric acid (final concentration, 4.5%). The soluble contents of each well were removed and neutralized with 1.0 M KOH. After centrifugation for 5 min at 10,000 × *g*, inositol phosphates were isolated by column chromatography. Supernatants were loaded onto columns (0.7 cm × 1.5 cm) of Bio-Rad AG 1-X8 resin (200–400 mesh, formate form) and, after being washed twice with 5 ml of water and twice with 5 ml of buffer containing 5 mM disodium tetraborate and 60 mM sodium formate, fractions containing inositol phosphates were eluted with 2 × 3.5 ml of 0.1 M formic acid/0.2 M ammonium formate. Radioactivity was determined with Ecolite scintillation fluid. Radioactivity in membrane phospholipids was calculated as the sum of radioactivity in the pellet formed after the addition of KOH and radioactivity solubilized with 0.1 N NaOH from cell membranes remaining in the wells. Data were expressed as the percentage of membrane phospholipids converted to inositol phosphates.

Statistics. All experimental values are reported as means ± standard errors. Comparisons of the data were made using a one-way analysis of variance conducted with StatView software for the Macintosh. Curve-fitting of competition data from binding experiments, including calculation of IC_{50} values, was carried out with SigmaPlot Scientific Graph System software for the Macintosh.

Results

In many systems a decrease in the affinity of a receptor for agonists is seen when assays are carried out in the presence of GTP. The affinity of 5-HT_2 receptors for ^{125}I-LSD was not affected by GTP (Fig. 1), suggesting that ^{125}I-LSD is either an antagonist or a weak partial agonist at these receptors. Based on this finding, it was possible to measure the effects of GTP on the inhibition of ^{125}I-LSD binding by 5-HT, LSD, and DOI as an index of the agonist activity of these compounds. In the absence of GTP, inhibition by LSD and DOI of ^{125}I-LSD binding was best fit by a single-site model with IC_{50} values of

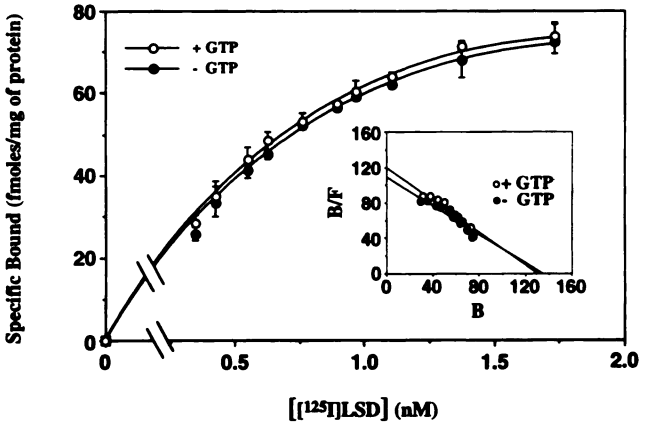

Fig. 1. Effects of GTP on the binding of ^{125}I-LSD to 5-HT_2 receptors on P11 cells. Membranes were incubated with increasing concentrations of ^{125}I-LSD in the presence or absence of 300 μM GTP. Ligand concentrations are plotted as a function of specific binding of radioligand. *Inset*, Scatchard transformation of the same data. *B*, Bound (fmol/mg of protein); *B/F*, bound/free (fmol/mg of protein/nM). Data shown are means ± standard errors of three determinations performed in triplicate.

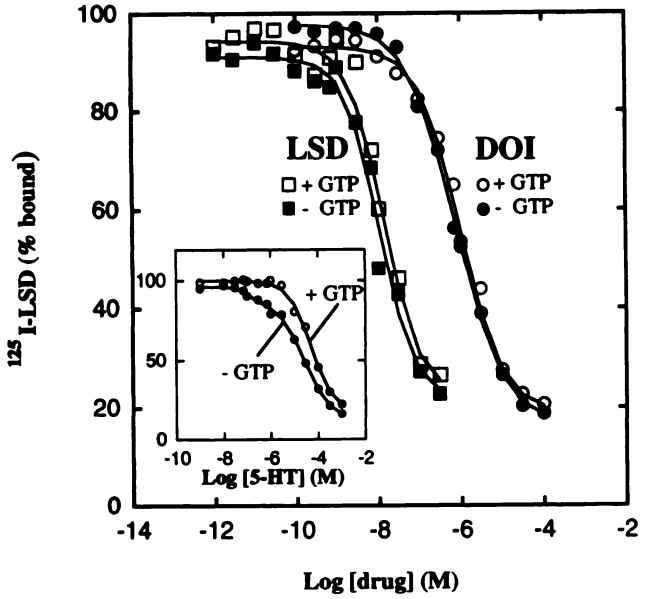

Fig. 2. Effects of GTP on inhibition by 5-HT, LSD, and DOI of ^{125}I-LSD binding to P11 cell membranes. The binding of 0.5 nM ^{125}I-LSD to receptors on P11 cell membranes was assayed with increasing concentrations of drug in the presence or absence of 300 μM GTP. *Inset*, effect of GTP on the inhibition by 5-HT of ^{125}I-LSD binding. Data shown are means of three determinations performed in triplicate.

10.4 nM and 737 nM, respectively (Fig. 2), whereas two sites, with high (IC_{50} = 134 nM) and low (IC_{50} = 24.8 μM) affinities for 5-HT, were seen (Fig. 2, *inset*). The presence of GTP had no detectable effect on competition curves with LSD or DOI (Fig. 2), suggesting that LSD and DOI do not induce formation of a significant amount of a ternary complex composed of agonist, receptor, and GTP-binding protein.

Drug-induced PI turnover was measured as a second indicator of agonist activity at 5-HT_2 receptors. Exposure to LSD, DOI, or 5-HT stimulated the production of inositol phosphates (Fig. 3). The largest increase in PI hydrolysis (350% over basal) was seen with 10 μM 5-HT, which was defined as a full agonist. Concentrations of LSD greater than or equal to 100 nM were able to elicit small but significant increases in PI hydrolysis,

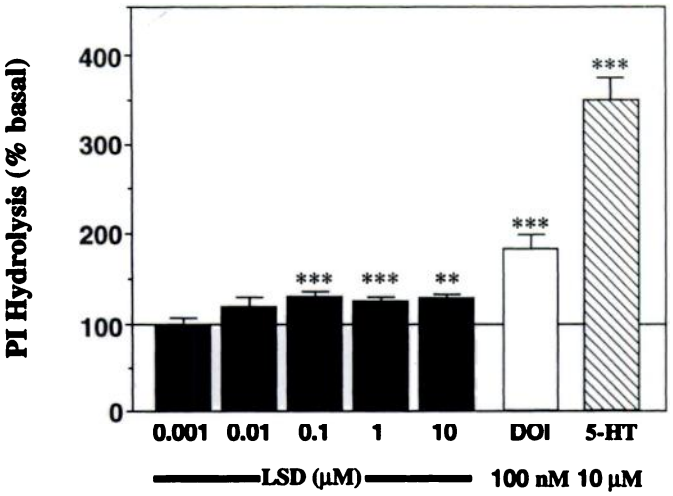

Fig. 3. Stimulation by LSD, DOI, or 5-HT of PI hydrolysis in P11 cells. Cells were incubated for 40 min with various concentrations of LSD, DOI, or 5-HT in the presence of 20 mM LiCl, and the ability of these compounds to elicit PI turnover was measured. Data shown are means ± standard errors of three to six determinations. Similar results have been obtained in two additional experiments. ***, $p < 0.001$, versus basal; **, $p < 0.01$.

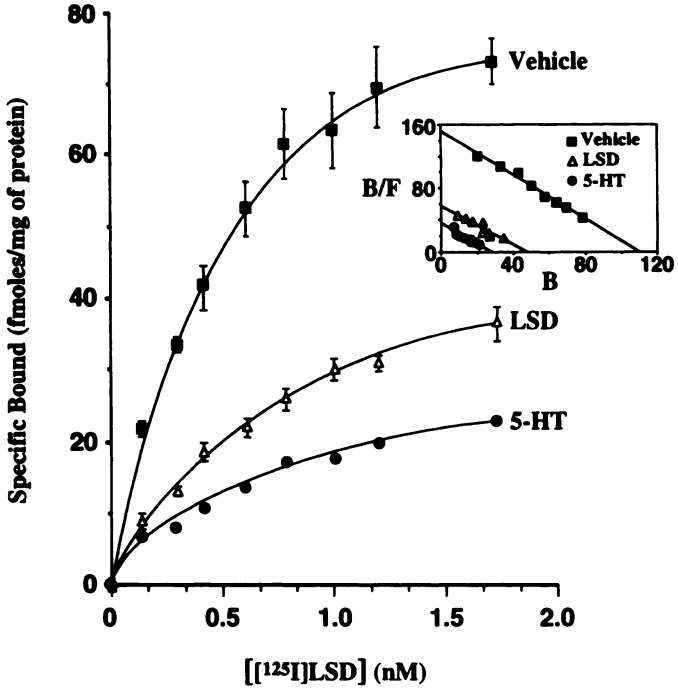

Fig. 4. Effects of LSD and 5-HT on the regulation of 5-HT$_2$ receptors on P11 cells. Cells were grown for 5 days and treated overnight with 1 μM LSD or 10 μM 5-HT. Membranes were then incubated with increasing concentrations of ^{125}I-LSD. The data shown represent means ± standard errors of four determinations, each assayed in triplicate. *Inset*, mean data from saturation isotherms for each treatment group transformed by the method of Scatchard. *B*, Bound (fmol/mg of protein); *B/F*, bound/free (fmol/mg of protein/nM).

with a maximum stimulation of 25% over basal levels. The efficacy of LSD was <10% of that of 5-HT. LSD-stimulated PI turnover was not due to stimulation of α_1-adrenergic receptors known to be present on P11 cells, because 1 μM prazosin did not affect the ability of LSD to stimulate PI hydrolysis (data not shown).

DOI exhibited weak partial agonist activity at 5-HT$_2$ receptors on P11 cells (Fig. 3). However, DOI was more efficacious (79% increase over basal at a concentration of 100 nM) than LSD. The efficacy of DOI was approximately 25% of that seen with 5-HT. Ketanserin and mianserin were also tested for their ability to stimulate PI hydrolysis in P11 cells. Ketanserin (1 μM) and mianserin (1 μM) did not stimulate PI turnover in P11 cells but completely prevented 5-HT-stimulated PI hydrolysis, consistent with these compounds being antagonists at 5-HT$_2$ receptors (data not shown).

Administration of LSD to rats results in a decrease in the density of 5-HT$_2$ receptors in various regions of the brain (11, 12). The ability of LSD and 5-HT to regulate 5-HT$_2$ receptors in P11 cells was investigated. Saturation analysis of ^{125}I-LSD binding to membranes isolated from cells treated with 1 μM LSD or 10 μM 5-HT for 16 hr demonstrated that LSD and 5-HT caused reductions in the density of 5-HT$_2$ receptors (Fig. 4), without altering the K_d of the radioligand for the receptors (Fig. 4, *inset*). Exposure of P11 cells to LSD reduced the density of 5-HT$_2$ receptors by approximately 50%, whereas exposure to 5-HT reduced the density of receptors by approximately 75% (Table 1). The effects of 5-HT and LSD on the density of 5-HT$_2$ receptors were prevented by coincubation with 1 μM ketanserin (Table 2).

When P11 cells were incubated for 16 hr with DOI, a compound shown to down-regulate 5-HT$_2$ receptors *in vivo* (20, 21), a marked reduction in the density of 5-HT$_2$ receptors was also observed (Table 1). DOI reduced the density of 5-HT$_2$ receptors by approximately 50%, equivalent to the magnitude of down-regulation elicited by 1 μM LSD. The affinity of the receptors for ^{125}I-LSD was unaffected. The reduction in the density of 5-HT$_2$ receptors after exposure to 100 nM DOI was prevented by 1 μM ketanserin (Table 2).

TABLE 1

Down-regulation of 5-HT$_2$ receptors on P11 cells after treatment with LSD, DOI, or 5-HT

P11 cells were grown to confluence, incubated with drug for 16 hr, and harvested as described previously (37). ^{125}I-LSD binding to 5-HT$_2$ receptors in membrane homogenates was then carried out using radioligand concentrations ranging from 0.2 to 2.0 nM. Saturation data thus generated were transformed by the method of Scatchard, and K_d and B_{max} values were calculated. Values shown are means ± standard errors from two independent experiments (four or more determinations).

Treatment	K_d	B_{max}
	nM	*fmol/mg of protein*
Vehicle	0.87 ± 0.07	103.9 ± 12.2
LSD (1 μM)	1.05 ± 0.12	49.4 ± 1.6*
DOI (100 nM)	1.04 ± 0.15	47.8 ± 6.7*
5-HT (10 μM)	0.98 ± 0.16	26.3 ± 1.5*

*$p < 0.001$, versus vehicle.

Down-regulation of receptors after exposure to LSD, DOI, or 5-HT was time dependent (Fig. 5). The maximal decrease in the density of receptors after administration of LSD, DOI, or 5-HT occurred within 4–8 hr. The extent of down-regulation of receptors was greater with 5-HT than with LSD or DOI, although a similar rate of decrease in the density of receptors was observed (Fig. 5). Exposure to a lower concentration of DOI (100 nM) caused a 50% reduction in receptor levels, similar to that seen after treatment of cells with 5 μM DOI (data not shown).

Slowly reversible or irreversible binding of LSD or DOI to 5-HT$_2$ receptors on P11 cells would lead to an apparent decrease in the density of receptors. To investigate this possibility, P11

cells were treated with 1 μM LSD or 100 nM DOI for 30 min, after which membranes were isolated and Scatchard analysis of ^{125}I-LSD binding was carried out. This period of treatment is too short for a significant down-regulation of 5-HT$_2$ receptors to occur, but is sufficient for the binding of these drugs at the 5-HT$_2$ receptor to reach equilibrium. In neither case was the density of 5-HT$_2$ receptors or their affinity for ^{125}I-LSD altered (Table 3). This result is consistent with a fully reversible interaction between LSD or DOI and 5-HT$_2$ receptors and indicates that the decrease in the number of 5-HT$_2$ receptors observed after longer periods of exposure is not due to irreversible or slowly reversible binding of these drugs to the receptors.

TABLE 2

Blockade of agonist-induced down-regulation of 5-HT$_2$ receptors by ketanserin

P11 cells were grown to confluence and incubated with or without 1 μM ketanserin for 15 min before the addition of 5-HT, LSD, or DOI. After a 5-hr incubation with drugs, cells were harvested and radioligand binding was carried out as described in Experimental Procedures. Values shown were calculated as a percentage of control radioligand binding and are reported as means ± standard errors of four to six determinations performed in triplicate.

Treatment	Receptors remaining	
	−Ketanserin	+Ketanserin
	% of control	
Vehicle	100.0 ± 4.6	105.9 ± 3.6
5-HT (10 μM)	38.5 ± 4.1[a]	96.0 ± 0.7
LSD (1 μM)	73.9 ± 3.7[b]	99.2 ± 0.4
DOI (100 nM)	68.5 ± 4.8[b]	103.1 ± 2.3

[a] $p < 0.001$, versus vehicle.
[b] $p < 0.005$.

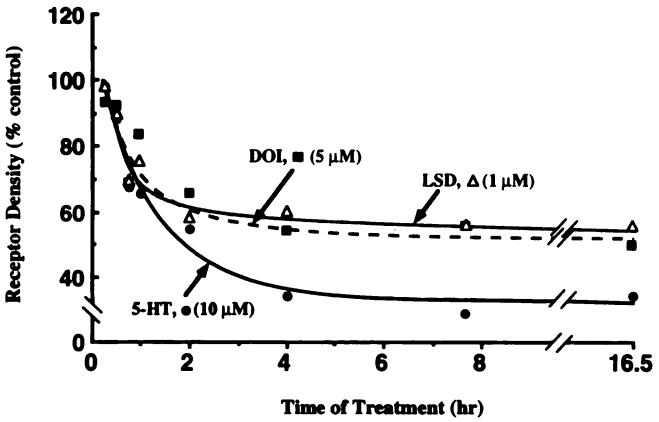

Fig. 5. Time course of down-regulation of 5-HT$_2$ receptors after exposure to agonists and partial agonists. P11 cells were treated with drugs for the indicated times, membranes were harvested, and binding assays were carried out with 0.2 and 1.0 nM ^{125}I-LSD, as described in Experimental Procedures. Results were calculated as a percentage of control values of vehicle-treated membranes and are shown as means of four determinations, each assayed in triplicate. Calculated simple first-order rate constants for receptor disappearance after administration of drugs were similar, ranging from 0.6 to 0.75 hr^{-1}.

TABLE 3

Effect of acute treatment of P11 cells with LSD or DOI on ^{125}I-LSD binding to 5-HT$_2$ receptors

P11 cells were grown to confluence, incubated with drug for 30 min, and harvested. Saturation binding data were transformed by the method of Scatchard, and K_d and B_{max} values were calculated. Values shown are means ± standard errors of three determinations performed in triplicate.

Treatment	K_d	B_{max}
	nM	fmol/mg of protein
Vehicle	0.87 ± 0.03	141 ± 6
LSD (1 μM)	1.13 ± 0.16	129 ± 7
DOI (100 nM)	1.02 ± 0.07	135 ± 4

The regulation of 5-HT$_2$ receptors by antagonists, which paradoxically decrease the density of 5-HT$_2$ receptors *in vivo* (29–33), was investigated. P11 cells were treated with vehicle, 1 μM ketanserin, 1 μM mianserin, or 10 μM 5-HT (as a positive control). High concentrations of antagonists (>100 × K_d) were chosen to ensure quantitative occupancy of 5-HT$_2$ receptors. Scatchard analysis of the binding of ^{125}I-LSD revealed that neither ketanserin nor mianserin altered the density of 5-HT$_2$ receptors after a 6- or 20-hr incubation with drug (Fig. 6). 5-HT, as expected, caused a marked reduction in the density of 5-HT$_2$ receptors. No change in the affinity of the receptors for the radioligand was observed, suggesting complete removal of residual drugs.

The observed lack of change in receptor density after exposure to antagonists could result from insufficient time of exposure to drugs or from uptake or metabolism of drugs. To investigate these possibilities, P11 cells were treated with mianserin for 72 hr. Neither the density of 5-HT$_2$ receptors nor the affinity of the receptors for the radioligand was affected by a 72-hr incubation with a high concentration of mianserin (Fig. 7). As a control for the stability and continued presence of mianserin during this extended incubation, cells were treated with 5-HT for the final 4 hr of a 72 hr exposure to mianserin (Fig. 7). The addition of 10 μM 5-HT to vehicle-treated cells led to an 80% reduction in the density of 5-HT$_2$ receptors. However, 10 μM 5-HT did not lead to a decrease in the density of 5-HT$_2$ receptors when added to cells treated with mianserin for 72 hr. This result indicates that even after a prolonged incubation mianserin is still present at a concentration that is sufficient to completely antagonize 5-HT-mediated down-regulation of the receptors.

Discussion

Most studies of the regulation of 5-HT$_2$ receptors have involved acute or chronic administration of drugs to laboratory

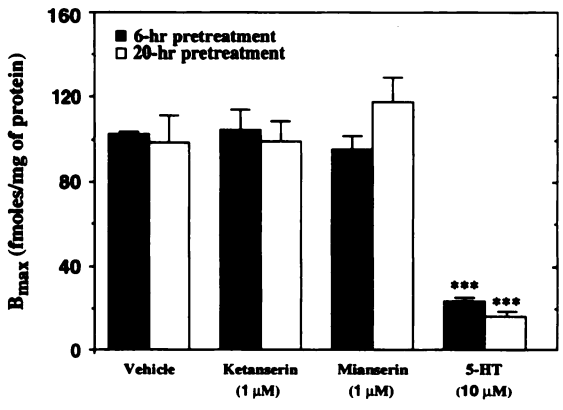

Fig. 6. Lack of regulation of 5-HT$_2$ receptors on P11 cells after treatment with antagonists. Cells grown for 5 days were treated with the 5-HT$_2$ receptor antagonists ketanserin and mianserin for either 6 or 20 hr and membranes were harvested using an extended washing protocol, as described in Experimental Procedures. Cells treated in parallel with 5-HT for the same periods of time were harvested in an identical manner and served as a positive control. B_{max} values reported are means ± standard errors of three or more experiments assayed on separate days. ***, $p < 0.001$, versus vehicle.

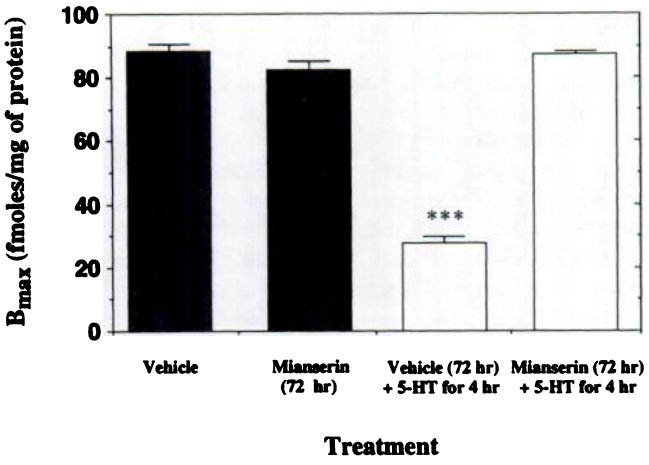

Fig. 7. Lack of regulation of 5-HT$_2$ receptors on P11 cells after prolonged incubation with mianserin. P11 cells were incubated with vehicle or 1 μM mianserin for 72 hr. Membranes were washed as described in Experimental Procedures and Scatchard analysis of the binding of ^{125}I-LSD was performed. Cells from additional experimental groups run in parallel were treated with vehicle or 1 μM mianserin for 72 hr. Four hours before harvesting (at 68 hr), 10 μM 5-HT was added to cells in both groups. B_{max} values reported are means ± standard errors of three determinations, each assayed in triplicate.***, $p < 0.001$, versus vehicle.

animals. However, the existence of interconnected neuronal networks and feedback mechanisms, which make it unlikely that one aspect of a specific neurotransmitter system can be manipulated without inducing secondary changes, may complicate the interpretation of results of *in vivo* studies. Paradoxical changes in the density of 5-HT$_2$ receptors after the administration of antagonists may be a consequence of complexities inherent in the use of *in vivo* models. *In vitro* model systems are useful for the study of drug interactions with receptors because variables that may hinder interpretation of the results of studies in animals (including drug disposition, drug metabolism, interacting neurons, and multiple receptor subtypes) are not present, or are more easily controlled, in cell culture. The recently developed P11 cell line (37), which expresses a high density of 5-HT$_2$ receptors coupled to PI hydrolysis, was chosen for examination of the effects of agonists and antagonists at 5-HT$_2$ receptors.

The ability of both agonists and antagonists of 5-HT$_2$ receptors to cause decreases in the density of 5-HT$_2$ receptors *in vivo* has raised questions concerning the efficacy of these drugs at the receptors. Effects of guanine nucleotides on radioligand binding and agonist inhibition of radioligand binding were investigated to identify agonist activity of these compounds. GTP did not alter the binding of ^{125}I-LSD to 5-HT$_2$ receptors in P11 cells. Because the binding of ^{125}I-LSD was insensitive to GTP, ^{125}I-LSD appeared to act as an antagonist radioligand in this system. This finding is similar to descriptions of the binding of [^{3}H]ketanserin (42) and [^{3}H]LSD (43) to 5-HT$_2$ receptors in rat frontal cortex. The lack of effect of GTP on the binding of ^{125}I-LSD to 5-HT$_2$ receptors allowed GTP to be included during membrane preparation to facilitate removal of residual drug and permitted investigation of the effects of GTP on agonist competition for ^{125}I-LSD-labeled sites. Inhibition by LSD and DOI of ^{125}I-LSD binding in the presence and absence of GTP did not reveal guanine nucleotide-sensitive effects. Peroutka *et al.* (43) reported a similar finding for inhibition by LSD of the binding of [^{3}H]LSD and [^{3}H]spiroperidol in rat cortical membranes. Effects of GTP on DOI inhibition of ^{125}I-LSD binding have not been investigated previously. The inability to detect effects of GTP on LSD or DOI binding to 5-HT$_2$ receptors suggests that these compounds are weak partial agonists or antagonists at 5-HT$_2$ receptors. In contrast, inhibition by 5-HT of the binding of ^{125}I-LSD was sensitive to GTP, an effect that has been observed using [^{3}H]ketanserin in rat cortical tissue (42).

Drug-induced turnover of PI in P11 cells was measured as a second index of agonist activity. Ketanserin (1 μM) and mianserin (1 μM) did not stimulate PI turnover in P11 cells but completely prevented 5-HT-stimulated PI hydrolysis. These findings demonstrate that stimulation of PI turnover by 5-HT is the result of a specific interaction with 5-HT$_2$ receptors and indicate that ketanserin and mianserin are full antagonists at this receptor. LSD and DOI were potent stimulators of PI hydrolysis. The efficacy of LSD, however, was <10% of that of the full agonist 5-HT, whereas DOI at a concentration of 100 nM had an efficacy of approximately 25%. Weak partial agonist activity is consistent with the effects of LSD on platelet activation (44), in calf coronary arteries (45), and in cortical slice preparations (14) and may explain the inability to detect GTP shifts in competition studies with LSD and DOI (see above).

In P11 cells, LSD and DOI caused down-regulation of 5-HT$_2$ receptors to nearly as great an extent as did the full agonist 5-HT. These effects were blocked by the prior addition of ketanserin, confirming that the observed effects were mediated by a specific interaction with 5-HT$_2$ receptors. The rate of down-regulation of receptors was similar after exposure to high concentrations of LSD, DOI, or 5-HT. The similarity in the kinetics of down-regulation of receptors after exposure to these drugs suggests that agonists and partial agonists cause down-regulation of receptors via similar mechanisms. Treatment of cells with 100 nM DOI, a concentration 7-fold less than the K_d of the receptors for this drug, caused the same maximal decrease in the density of receptors as did 5 μM DOI, indicating that extensive down-regulation of receptors can occur under conditions where few receptors are occupied by drug at any given time.

The presence of residual drug was not responsible for the observed changes in the density of receptors, because they were not accompanied by changes in the affinity of the receptors for the radioligand. In addition, irreversible drug binding is an unlikely explanation for the reduction in the density of 5-HT$_2$ receptors, because acute drug treatment did not alter B_{max} or K_d values. Together with the findings described above, the current data suggest that full and partial agonists of 5-HT$_2$ receptors elicit down-regulation of receptors by a specific interaction with the receptors, and they indicate that down-regulation of receptors by LSD, DOI, and 5-HT proceeds through common mechanisms.

The ability of LSD and DOI to down-regulate 5-HT$_2$ receptors in P11 cells was not proportional to the level of PI stimulation. LSD (1 μM), which produces a 25% increase in PI hydrolysis over basal levels, and DOI (100 nM), which causes a 79% increase in PI turnover, both caused similar reductions in the density of 5-HT$_2$ receptors in P11 cells and were nearly as efficacious as the full agonist 5-HT. Consequently, the degree to which these drugs stimulate PI hydrolysis in P11 cells is not a reliable indicator of their ability to down-regulate 5-HT$_2$ receptors. One interpretation of these results is that limited

stimulation of PI hydrolysis is sufficient to activate processes controlling receptor down-regulation. Alternatively, the discrepancy between the magnitude of PI hydrolysis and the extent of down-regulation could indicate that these two processes are independent and that other intracellular signals are involved in the down-regulation of 5-HT_2 receptors.

As mentioned previously, chronic administration of antagonists *in vivo* unexpectedly leads to marked decreases in the density of 5-HT_2 receptors (29–33). The possibility that novel regulatory mechanisms are responsible for the observed changes has been considered. Nonetheless, interpretation of the anomalous effects of antagonists have focused on more easily explained possibilities such as residual drug, increased synaptic levels of 5-HT, and interactions with other neurotransmitter systems (34, 46). In studies of the effects of antagonists, treatment of cultured calf aorta smooth muscle cells with ketanserin or setoperone decreased 5-HT-induced inositol phosphate formation (28). The authors suggested that desensitization could be explained by antagonist-induced down-regulation of 5-HT_2 receptors, an explanation in accordance with *in vivo* regulation of 5-HT_2 receptors by antagonists. However, antagonist-induced desensitization of 5-HT_2 receptor responses in these cells may also have been due to the presence of residual drug (35). The low density of 5-HT_2 receptors on these cells precluded the use of radioligands to directly measure effects on receptor number.

P11 cells were used to investigate antagonist regulation of the density of 5-HT_2 receptors because a high density of receptors is expressed on this clonal cell line. In addition, treatment of P11 cells with agonists revealed that 5-HT_2 receptors on these cells respond to drug challenges by regulating receptor density. P11 cells were treated with high concentrations of mianserin and ketanserin, compounds that behaved as full antagonists in studies of the stimulation of PI hydrolysis. Pilot experiments revealed that mianserin and ketanserin were difficult to remove from P11 cell membranes, as shown by a decrease in the apparent affinity of the receptors for ^{125}I-LSD (data not shown). Residual drug was removed, however, by use of an extended membrane harvesting and washing protocol. Scatchard analysis revealed that treatment of P11 cells with ketanserin or mianserin did not alter the density or properties of 5-HT_2 receptors, in contrast to the observed regulation of 5-HT_2 receptor density after antagonist administration *in vivo*. Prolonged exposure to mianserin, in a paradigm that simulates chronic administration of antagonists *in vivo*, also failed to regulate the density of 5-HT_2 receptors on P11 cells, further indicating that antagonists will not directly alter receptor expression. Antagonism by mianserin of 5-HT-mediated down-regulation of 5-HT_2 receptors demonstrated that the inability of mianserin to regulate the density of receptors was not a consequence of instability or uptake of mianserin. The inability of antagonists to regulate 5-HT_2 receptors in an isolated cell culture system, where indirect actions of drugs are limited or nonexistent, suggests that the effects observed *in vivo* cannot be due to a direct interaction of the drugs with the receptors and indicates that agonists and antagonists may decrease receptor density through different mechanisms.

It has been suggested that regulation of 5-HT_2 receptors by antagonists may occur via a mechanism that differs from that of agonists. Leysen and Pauwels (28) investigated regulation of 5-HT_2 receptors *in vivo* by the agonist DOM and the antagonist ketanserin. The effects of agonist, antagonist, or a combination of agonist and antagonist were measured. The magnitude of down-regulation produced by coincubation with agonist and antagonist (31%) was midway between the effects of agonist (40%) and antagonist (19%) alone. The authors concluded that the antagonist partially prevented the down-regulation by the agonist, but this effect was masked by the down-regulation produced by the antagonist. Studies with calf aorta smooth muscle cells demonstrated agonist (DOM)-induced, but not antagonist (cinanserin)-induced, desensitization of responses mediated by 5-HT_2 receptors (35). Based on this difference, the authors postulated that regulation by antagonists *in vivo* may be a heterologous process due to release of mediators triggered by the action of antagonists. Data from our studies on P11 cells are consistent with this conclusion.

In summary, this report provides information on the ability of agonists and antagonists to regulate the density of 5-HT_2 receptors *in vitro*. Previous studies of drug-induced regulation of 5-HT_2 receptors were carried out *in vivo*, where agonists and antagonists were shown to be similarly capable of down-regulating 5-HT_2 receptors. In the current study, agonists, but not antagonists, caused a decrease in the density of 5-HT_2 receptors. It is hypothesized that agonists and antagonists of 5-HT_2 receptors regulate the density of receptors by distinct mechanisms *in vivo*, presumably via direct and indirect actions, respectively.

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