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Antagonist-Mediated Down-regulation of 5-Hydroxytryptamine Type 2 Receptor Gene Expression: Modulation of Transcription

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SUMMARY

Prolonged exposure to an agonist results in a progressive loss of most G protein-coupled receptors, whereas exposure to an antagonist causes increased receptor response. The 5-hydroxytryptamine (5-HT)$_2$ receptor is down-regulated by agonists but, paradoxically, antagonists can also elicit a decrease in receptor density. Here we show that long term treatment with serotonin or mianserin, an antagonist and antidepressant, results in reduced levels of both the 5-HT$_2$ receptor and its RNA. Antagonist-induced down-regulation requires the presence of the 5-HT$_2$ receptor, it occurs at the level of transcription, and it is mediated by a drug response sequence in the 5′ flanking region of the 5-HT$_2$ receptor gene. The effect of mianserin might result, at least in part, from its ability to modulate transcription.

For most G protein-linked receptors, agonist stimulation can initiate desensitization, a process that causes them to become progressively less able to trigger signaling mechanisms (1). The molecular mechanisms underlying desensitization, which is essentially complete within minutes, include phosphorylation and sequestration of the receptor (2). More prolonged exposure to an agonist can result in a progressive reduction in receptor density, and this may be associated with a decrease in the level of receptor mRNA (3, 4). In contrast, with loss of presynaptic input or chronic treatment with antagonists receptors develop disuse supersensitivity, a state characterized by an increase in receptor density (5).

The 5-HT$_2$ receptor mediates many of the physiological functions of serotonin (5-HT). The 5-HT$_2$ receptor activates a G protein, which in turn stimulates phospholipase C-catalyzed hydrolysis of phospatidylinositol lipids to yield the second messengers inositol triphosphate and diacylglycerol, resulting in Ca^{2+} release from intracellular stores and activation of protein kinase C (6). Recently, a cDNA corresponding to the 5-HT$_2$ receptor has been cloned, facilitating its study at the molecular level (7, 8). Although the receptor exists in multiple functional states, no evidence has been found for multiple gene subtypes (9). The 5′ flanking region of the 5-HT$_2$ receptor gene has been cloned and sequenced, and its transcriptional regulatory functions have been analyzed (10). The promoter lacks an identifiable TATA motif and utilizes at least 11 clustered start sites.

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The 5-HT$_2$ receptor is down-regulated after administration of agonists such as LSD and amphetamine derivates (11–14). Similarly, long term treatment with monoamine oxidase inhibitors and 5-HT uptake blockers also reduces the receptor density. Paradoxically, however, long term administration of antagonists can also elicit a decrease in receptor density (15, 16). The down-regulation of the 5-HT$_2$ receptor by both agonists and antagonists has been demonstrated in human subjects and in animal experiments (11, 13–17).

Many drugs that down-regulate the 5-HT$_2$ receptor have anxiolytic and antidepressant effects (17, 18), including monoamine oxidase inhibitors, 5-HT uptake blockers, and 5-HT$_2$ receptor antagonists such as mianserin (19, 20). The development of the therapeutic and receptor-down-regulating effects of antidepressants requires long term treatment, indicating that slowly acting mechanisms, such as gene regulatory processes, form the basis for drug action.

In this report we demonstrate that extended treatment of cultured rat C6 glioma cells with both ligand and the antagonist mianserin causes a reduction in the level of the endogenous 5-HT$_2$ receptor and its mRNA. In contrast, only the antagonist, and not 5-HT, causes a similar reduction in the expression of an exogenous reporter gene, whose expression is controlled by the 5-HT$_2$ receptor promoter, suggesting that the antagonist-mediated down-regulation occurs at the level of transcription. This notion is further supported by the observation that the down-regulation is mediated by a specific DNA sequence, a mianserin response element, in the 5′ flanking region of the receptor gene. Because 5-HT does not significantly reduce the

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; LSD, lysergic acid diethylamide; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; kb, kilobase(s).

expression of the exogenous 5-HT_2 receptor-reporter gene construct, the ligand-induced receptor down-regulation is most likely not caused by a transcriptional effect. Thus, the mechanisms of ligand- and antagonist-mediated down-regulation of the 5-HT_2 receptor are distinct and probably involve different signaling pathways.

Materials and Methods

Cell culture and drug treatment. Rat C6 glioma cells (6) and rat CREF fibroblasts were grown in Dulbecco's modified minimal essential medium supplemented with 15% horse serum plus 2.5% fetal calf serum. C6 cells express the 5-HT_2 receptor (38 fmol/mg of membrane protein) (10). Mouse L and A9/6.2 cells were grown in medium containing 10% fetal calf serum. The A9/6.2 cell line (a gift from B. Hoffman, Laboratory of Cell Biology, National Institute of Mental Health) was produced by transfecting mouse L cells with a 5-HT_2 receptor cDNA in the expression vector pCD1, essentially as described (9). A9/6.2 cells stably express the 5-HT_2 receptor (120 fmol/mg of membrane protein). Cells were treated with 5-HT (Sigma Chemical Co., St. Louis, MO), mianserin, or ketanserin (Research Biochemicals Inc., Natick, MA) for either 2 or 6 days. Drug treatments were initiated when cell cultures were approximately 25% confluent; monolayers were essentially confluent by the time extracts for CAT assays or RNA were prepared. Cells were washed, incubated in fresh medium for 1 hr, and washed again before preparation of membranes or RNA.

Membrane preparation and drug binding assay. Total cell membranes were collected by centrifugation (17,000 × *g* for 20 min) in buffer containing 10 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$ (9). Binding assays were performed as described (21), in 50 mM Tris·HCl, pH 7.4, using 30 μg of membrane protein/0.1-ml volume and 1 nM ^{125}I-LSD. Nonspecific binding was defined with 1 μM mianserin.

RNA isolation and RNase protection experiments. RNA isolation used the guanidium thiocyanate-CsCl procedure (22). Probes for RNase protection assays were generated by *in vitro* transcription with T3 or SP6 RNA polymerases in the presence of [^{32}P]CTP (800 Ci/mmol). Rat 5-HT_2 RNA probe corresponding to nucleotides 1938–2170 of the cloned cDNA sequence (7) was synthesized with a plasmid containing the rat 5-HT_2 receptor cDNA cloned into pGEM-3 and linearized by *Nco*I. *Xba*I-cleaved pSP6-β-actin (Ambion) was used to generate the mouse β-actin probe. Probes were purified by polyacrylamide gel electrophoresis. For RNase protection assays, the annealing temperature was 45°, and digestion was carried out for 45 min at 30° with final concentrations of 2.5 units/ml RNase I and 100 units/ml RNase T_1. Protected fragments were separated by electrophoresis in 6% polyacrylamide gels containing 7 M urea. Bands corresponding to the protected fragments were cut from the gel and the radioactivity was measured.

Transient expression assays. Different segments of the 5′ flanking region of the 5-HT_2 receptor gene were linked to the bacterial CAT gene as described (10). Briefly, a 5.1-kb *Hind*III-*Hind*III fragment representing the entire available 5′ flanking region was fused to the CAT gene, generating plasmid 5-HT_2R-5.6CAT. The flanking region of the 5-HT_2 receptor gene was shortened by excising DNA fragments and religating the remaining portion of the plasmid. *Hind*III combined with *Xho*I, *Acc*I, and *Nsi*I generated 5-HT_2R-2.7CAT, 5-HT_2R-2.3CAT, and 5-HT_2R-1.5CAT, respectively. The *Nde*I-*Xho*I subfragment (between −2.2 and −2.7 kb) of the 5-HT_2 receptor 5′ flanking region or a synthetic AP-1 site was also fused to the TK-CAT gene (pBLCAT) (23).

To assay transient expression, 10 μg of plasmid DNA were used to transfect approximately 5 × 10^5 cells by the calcium phosphate-DNA coprecipitation method (24). Two hours after the beginning of transfection, mianserin or 5-HT was added to some of the cultures at a final concentration of 100 nM or 10 μM, respectively. After overnight incubation with the DNA precipitate, cells were washed and fresh medium containing drug was added. After a 2-day incubation, cells were collected, protein concentration was determined by the method of Bradford (25), using bovine serum albumin as a standard, and 200 μg of cell protein were assayed for CAT activity (26). The final concentration of coenzyme A was 8 mM in the enzymatic reaction, the incubation was continued for 3–4 hr at 37°, and reaction products were separated on thin layer chromatography silica plates (Baker). Bands corresponding to acetylated and nonacetylated [^{14}C]chloramphenicol were scraped from the plates, and radioactivity was quantified in a scintillation counter.

Results

The effects of 5-HT and mianserin on the 5-HT_2 receptor level were assayed using C6 glioma cells. These cells express the 5-HT_2 receptor, a conclusion based on pharmacological profiles of drug binding assays and on the fact that the cells contain 5-HT_2 receptor-specific RNA (10). No evidence was found for the presence of 5-HT receptors other than 5-HT_2 in these cells (see Discussion). C6 glioma cells were incubated in the presence of the drugs for 6 days and then assayed for ^{125}I-LSD binding (LSD binds to 5-HT_2 receptors with high affinity). The density of 5-HT_2 receptor binding sites was reduced by 1 or 10 μM 5-HT (42% and 37% of control, respectively) or 10 nM mianserin (39% of the control). Long (2–6-day) but not short (1-hr) treatment altered receptor density (data not shown), consistent with earlier work performed in animals (15, 16).

Treatment of C6 cells with 5-HT or mianserin (2 or 6 days) caused a 50–70% decrease in the level of 5-HT_2 receptor-specific RNA (Fig. 1, a and c). As a control, the level of β-actin RNA was monitored and found to be unchanged (Fig. 1a). The magnitude of the reduction in the 5-HT_2 receptor RNA level fits well with the decrease observed for receptor binding activity. The effect of 5-HT and mianserin on the level of 5-HT_2 receptor RNA was dose dependent. Although 1 μM 5-HT (K_d in the micromolar range) or 1 nM mianserin (K_d in the nanomolar range) was sufficient to reduce the level of RNA, concentrations of 10 μM 5-HT or 10–100 nM mianserin were more effective, reducing 5-HT_2 receptor RNA levels by a factor of 2–3 (Fig. 1, b and c). Higher doses (100 μM 5-HT and 1 μM mianserin) did not cause an additional reduction in RNA level (data not shown). Ketanserin, a 5-HT receptor antagonist that is highly selective for the 5-HT_2 receptor (27), also reduced the level of receptor RNA (Fig. 1b). Ketanserin, at least at higher drug concentrations, caused somewhat less reduction in RNA levels than did mianserin, suggesting that the receptor-down-regulating and antagonist effects of these drugs are not necessarily operating in parallel. In fact, the two activities are markedly different in terms of kinetics (see also Discussion).

The reduced steady state level of receptor-specific mRNA observed after mianserin treatment (Fig. 1) could be the result of RNA instability or lower transcriptional rate in treated cells. Fig. 2 shows that the reduced 5-HT_2 receptor RNA level in mianserin-treated cultures was not due to destabilization of mRNA. When new mRNA synthesis was blocked with actinomycin D, untreated and mianserin-treated cells showed the same rates of 5-HT_2 receptor RNA decay.

Next, assays were performed to test whether the reduced 5-HT_2 receptor RNA levels in mianserin-treated cells resulted from down-regulation of the receptor gene at the level of transcription. The activity of reporter plasmids, containing different portions of the 5′ flanking region from the 5-HT_2 receptor gene controlling expression of the CAT gene, was

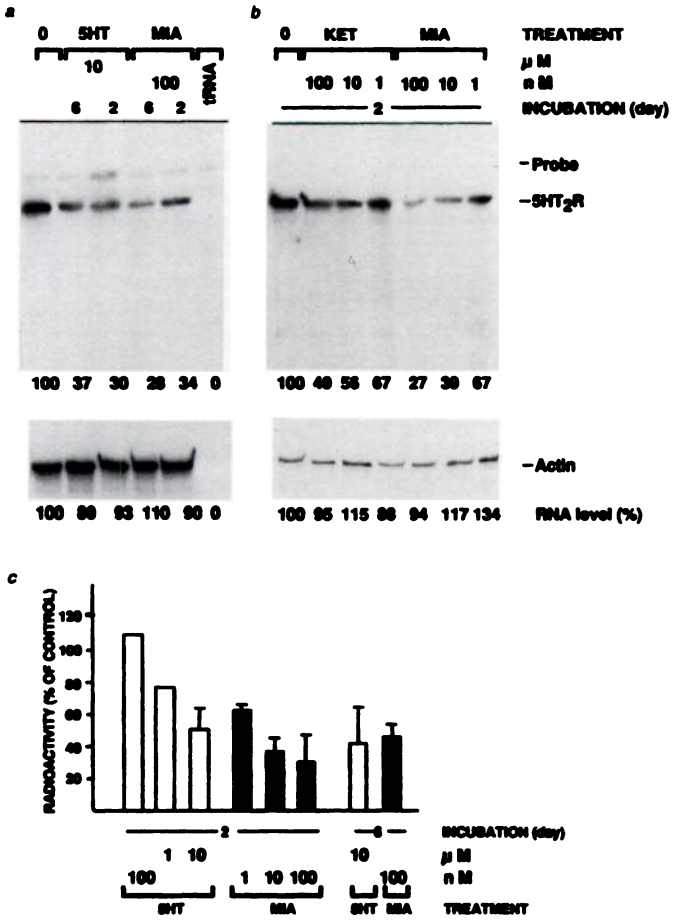

Fig. 1. 5-HT and mianserin (*MIA*) down-regulate the 5-HT$_2$ receptor RNA in C6 cells. a, Representative RNase protection assay with the 5-HT$_2$ receptor (*5HT$_2$R*) and β-actin probes, in cultures treated with 5-HT or mianserin for 2 or 6 days. b, Dose-dependent down-regulation of 5-HT$_2$ receptor RNA levels by treatment for 2 days with mianserin or ketanserin (*KET*). *Numbers under the lanes*, radioactivity of bands as a percentage of the control. c, Summary of multiple RNase protection experiments, showing the dose-dependent down-regulation of 5-HT$_2$ receptor RNA levels by 5-HT and mianserin.

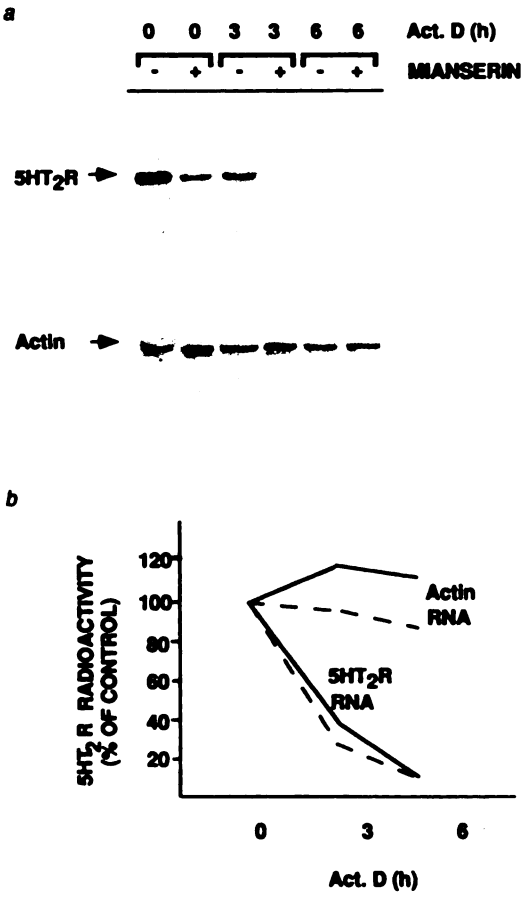

Fig. 2. Mianserin does not influence 5-HT$_2$ receptor (*5HT$_2$R*) mRNA stability. a, C6 cultures were treated with 100 nM mianserin for 2 days or left untreated, and then cultures were harvested for RNA isolation (*0 h*) or treated with actinomycin D (*Act. D*) (5 μg/ml) for 3 or 6 hr before RNA isolation. Other conditions were as in Fig. 1. b, Graphic representation of experimental results shown in a. *Lines*, decay of RNA levels in either mianserin-treated (– – –) or untreated (——) cultures.

measured in drug-treated and control cells (Fig. 3). Cells transfected with a control TK-CAT construct (CAT expression is directed by the herpes simplex TK gene promoter) showed no change in CAT activity upon mianserin treatment (Fig. 3a). In contrast, CAT activity was reduced in response to mianserin in cells transfected with plasmids containing portions of the 5-HT$_2$ receptor gene 5′ flanking sequence. CAT expression controlled by DNA segments extending upstream from the 5-HT$_2$ receptor translational initiation codon to −2.3 kb or −2.7 kb was inhibited by 35–60% (Fig. 3). A longer upstream sequence, extending to −5.6 kb, did not exhibit a greater response to mianserin (data not shown). Expression of CAT controlled by a 5-HT$_2$ receptor 5′ flanking DNA segment extending to −1.5 kb was not influenced by mianserin treatment (Fig. 3b). These experiments located a mianserin response sequence within the region between −1.5 kb and −2.7 kb upstream of the 5-HT$_2$ receptor translational initiation codon. Importantly, the extent of down-regulation of the exogenous 5-HT$_2$ receptor-CAT reporter gene was comparable to that of the endogenous receptor gene and its mRNA in mianserin-treated cells (Figs. 1 and 2), suggesting that transcriptional regulation is most likely the predominant mechanism involved in the drug-induced receptor down-regulation.

Although agonist-mediated down-regulation of 5-HT receptors is an extensively studied phenomenon, it is not known whether transcriptional mechanisms are involved in this process. To answer this question, CAT plasmids containing portions of the 5-HT$_2$ receptor gene 5′ flanking sequence were transfected into C6 cells and enzyme activities were determined in cultures incubated in the presence or absence of 5-HT (Fig. 3). 5-HT treatment caused little if any reduction in CAT activity, in sharp contrast to the down-regulation observed in the levels of the endogenous receptor and its mRNA (Figs. 1 and 3). Similarly, no 5-HT effect was found using a longer upstream sequence, extending to −5.6 kb of the 5′ flanking region of the receptor gene (data not shown), suggesting either that transcriptional regulation is not the primary mechanism in the 5-HT-mediated receptor down-regulation or that the region involved in transcriptional control is outside of the cloned (−5.6 kb to −0.6 kb) receptor 5′ flanking sequence. In any event, these experiments demonstrated that the down-regulation induced by mianserin is distinct from that mediated by 5-HT, in that the former is mediated by a mianserin response region located between −1.5 kb and −2.7 kb in the 5′ flanking region

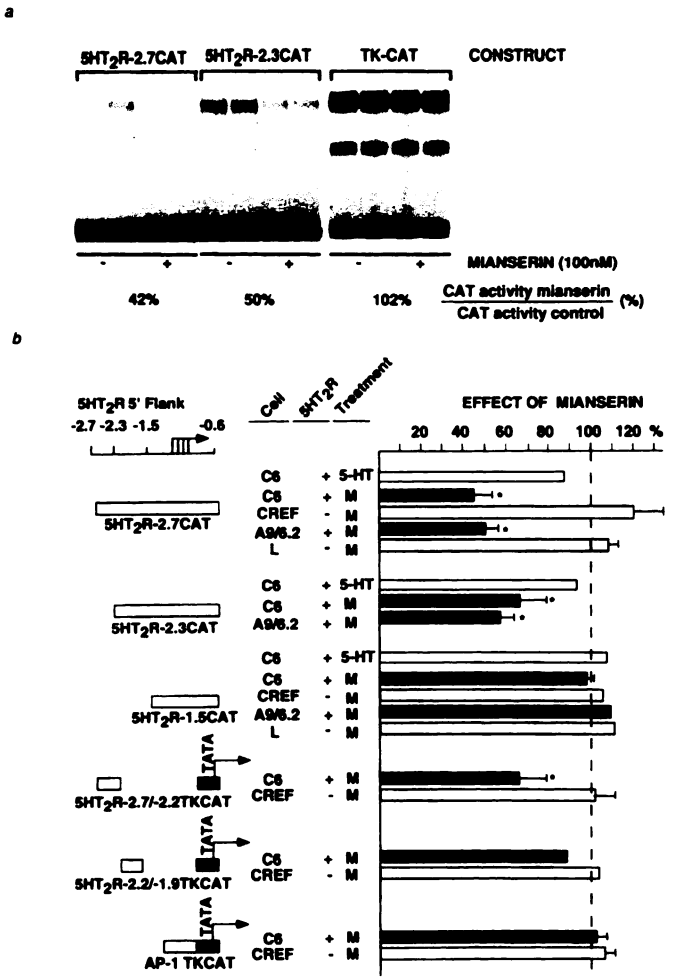

Fig. 3. The 5′ flanking region of the 5-HT_2 receptor gene is involved in mianserin-mediated down-regulation of gene expression. a, Representative CAT assays of duplicate C6 cell cultures transfected with reporter constructs containing upstream sequences extending to −2.7 and −2.3 kb (relative to the AUG codon) from the 5′ flanking region of the 5-HT_2 receptor (*5HT$_2$R*) gene or TK-CAT (5′ flanking region of the TK promoter), which served as a control. The TK promoter was 5–10-fold more active than the promoter of the 5-HT_2 receptor gene. b, Summary of the results of three or four independent experiments using constructs with different amounts of 5′ flanking region of the 5-HT_2 receptor gene upstream of the homologous or heterologous basal promoter. The effect of mianserin or 5-HT on CAT expression was calculated as shown in a but normalized to its effect in the control TK-CAT-transfected cultures. *Arrows*, transcription initiation sites in the 5-HT_2 receptor and TK promoters. C6 cells and the L cell derivative A9/6.2 express 5-HT_2 receptor; however, CREF and L cells do not express the receptor. *M*, mianserin treatment. *, Significant ($p < 0.05$, paired-samples *t* test) effect of mianserin on CAT enzyme activity.

of the 5-HT_2 receptor gene. To explore further the nature of the mianserin response sequence, we examined whether the upstream sequence of the 5-HT_2 receptor gene can mediate mianserin-induced transcriptional down-regulation in the context of a heterologous promoter.

The DNA sequence located between −1.5 kb and −2.2 kb, containing the basal promoter activity of the 5-HT_2 receptor gene (10), was replaced by the heterologous TK promoter fragment, resulting in hybrid promoters consisting of the 5-HT_2 receptor 5′ flanking DNA segment from −2.7 kb to −2.2 kb or from −2.2 kb to −1.9 kb appended to the TK sequence. The TK promoter, in contrast to the 5-HT_2 receptor promoter, contains a single initiation site and a consensus TATA motif. Expression directed by the promoter fragment between −2.7 and −2.2 kb was reduced by mianserin, whereas expression from a construct containing 5-HT_2 receptor gene sequences from −2.2 kb to −1.9 kb or from a control construct with an AP-1 site cloned upstream of the same TK promoter was not affected (Fig. 3b). This experiment confirmed the existence of a mianserin response element, refined its location to the sequence between 2.2 kb and 2.7 kb upstream of the 5-HT_2 receptor translational initiation codon, and demonstrated that the function of the response region is not dependent on a particular mode of transcriptional initiation.

The fact that micromolar levels of the natural ligand or nanomolar quantities of antagonists (including the highly selective drug ketanserin) down-regulated the level of 5-HT_2 receptor RNA (Fig. 1) is consistent with the notion that binding to the 5-HT_2 receptor itself is required to mediate the effect. Direct evidence for a role of the 5-HT_2 receptor in down-regulation by mianserin was obtained using the A9/6.2 cell line, which expresses the receptor, and its parental mouse L cell line, which lacks receptor expression. Mianserin reduced expression of CAT reporter constructs that included the drug response sequence in A9/6.2 cells, but not in the parental L cells (Fig. 3b), indicating that the effect of mianserin on transcription requires the presence of the 5-HT_2 receptor.

Discussion

Long term treatment with 5-HT or mianserin, a 5-HT antagonist and antidepressant, causes a reduction in the level of 5-HT_2 receptors (measured by ^{125}I-LSD binding) in rat C6 glioma cells, demonstrating that the regulation of this receptor type in this *in vitro* system properly mimics the *in vivo* situation. Although several 5-HT receptors bind ^{125}I-LSD with high affinity, the pharmacological profile of the receptor in C6 cells (10) indicates that these cells do not express 5-HT receptors other than 5-HT_2. Also, a 5-HT_2 receptor-specific antisense oligonucleotide down-regulated with equal efficiency both the ^{125}I-LSD binding and the 5-HT_2 receptor-specific mRNA level in C6 cells,[1] indicating again that the majority, if not all, of the 5-HT receptors in C6 cells are of the 5-HT_2 subtype.

5-HT and mianserin down-regulate not only the 5-HT_2 receptor but also its mRNA in rat C6 glioma cells. The magnitude of the reduction in 5-HT_2 receptor RNA level fits well with the decrease observed for receptor binding activity. In contrast to our results, Ferry *et al.* (28) recently reported that mianserin did not alter the density of 5-HT_2 receptors on P11 cells. These cells are a clonal cell line derived from a rat pituitary tumor, and it is possible that the 5-HT_2 receptor is not regulated in the same fashion in all cells. It is not known whether normal untransformed pituitary cells can respond to mianserin by down-regulation of the 5-HT_2 receptor level.

The major finding of this report is that mianserin down-regulates 5-HT_2 receptor gene expression at the level of transcription. As shown in Fig. 3, the activity of the receptor-reporter gene was down-regulated by mianserin. Because the extent of down-regulation of the exogenous 5-HT_2 receptor-reporter gene was similar to that of the endogenous receptor and its mRNA, the mianserin-mediated down-regulation of receptor is most likely achieved by a transcriptional mecha-

[1] M. Toth and D. Benjamin, unpublished observations.

nism. In contrast, 5-HT treatment resulted in little if any reduction in the activity of the exogenous receptor-CAT construct, suggesting either that transcriptional regulation is not the primary mechanism in 5-HT-mediated receptor down-regulation or that the region involved in transcriptional control is outside of the cloned (−5.6 kb to −0.6 kb) 5′ flanking receptor sequence. Thus, the transient expression experiments clearly demonstrate that mianserin and 5-HT induce down-regulation of the 5-HT$_2$ receptor by different mechanisms. Additional experiments defined a mianserin response sequence between −2.7 kb and −2.2 kb in the 5′ flanking region of the receptor gene (Fig. 3b). Interestingly, this sequence mediated the mianserin response in the context of the heterologous TK promoter, demonstrating that function of the response region is not dependent on a particular mode of transcriptional initiation.

Mianserin is an antagonist, and it effectively blocks signaling of the 5-HT$_2$ receptor, including inhibition of ligand-induced increases in phosphatidylinositol turnover and Ca^{2+} mobilization (15, 16, 29). The expected response to such inhibition is disuse supersensitivity, resulting in up-regulation of the receptor, probably as the consequence of suspension of receptor autoregulatory mechanisms. However, mianserin and perhaps other antagonists induce the opposite effect on the 5-HT$_2$ receptor in C6 cells. Thus, the following question arises: how can an antagonist down-regulate its cognate receptor? There are at least two mechanisms that could explain this paradox. First, by uncoupling the ability of the receptor to activate its cognate G protein in response to 5-HT, antagonists may interrupt a basal, constitutive level, signaling process that helps to maintain a normal level of receptor gene transcription. C6 cells express the 5-HT transporter[2] and therefore can accumulate and possibly release serum-derived 5-HT, which could then interact with the 5-HT$_2$ receptor and positively regulate receptor transcription. This could, in theory, maintain a low basal level of receptor activation. Such a positive auto-regulation of the β_2-adrenergic receptor gene appears to occur through receptor-mediated stimulation of adenyl cyclase, with consequent activation of cAMP response element-binding protein and stimulation of receptor gene transcription (30). Antagonists of the 5-HT$_2$ receptor may interrupt the receptor-mediated, constitutive level signaling, resulting in a reduction of receptor expression. Prolonged 5-HT treatment may induce a similar uncoupling by desensitization. However, the different responses to 5-HT and mianserin of the exogenous receptor-reporter construct suggest that the pathway for mianserin effect is distinct from that of 5-HT. Second, a drug may inhibit a ligand-induced response by blocking a specific pathway (antagonist effect) and at the same time may trigger another response via a distinct signaling pathway (agonist-like effect). According to this hypothesis, mianserin has a dual effect on the 5-HT$_2$ receptor. The drug rapidly interferes with 5-HT binding at the receptor and blocks the ligand-induced increase in phosphatidylinositol turnover and Ca^{2+} mobilization. However, prolonged exposure of the receptor to mianserin may also induce an alternative pathway that results in the down-regulation of genes including the 5-HT$_2$ receptor gene. The different time courses of the rapid antagonist (minutes) and slow receptor-down-regulating (days) effects of mianserin support the notion that mianserin might modify different signaling pathways coupled to the 5-HT$_2$ receptor, depending on the length of the receptor-drug interaction.

As described earlier, transcription of the 5-HT$_2$ receptor gene begins to change only after long term treatment with mianserin. The time course for induction of a change in transcription appears consistent with the slowly acting antidepressant and anxiolytic effects of mianserin *in vivo*. The action of mianserin might not rest on antagonist effects at the level of synaptic transmission or the action of second messengers; rather, mianserin might function, at least in part, by altering gene expression.

The nature of the proposed mianserin-induced pathway through which 5-HT$_2$ receptor mRNA levels are reduced is not known. We have analyzed the DNA sequence in the mianserin response region to identify known transcription factor binding sites, which in turn might provide insight into the corresponding signaling pathway. The best studied example of a neurotransmitter-regulated transcription factor is the cAMP response element-binding protein, which can also be modified by a Ca^{2+} signal (31). The mianserin response sequence of the 5-HT$_2$ receptor gene, however, does not contain a recognition site for the cAMP response element-binding protein. We have found several AP-1 sites in the mianserin response sequence, but because no response to the drug was obtained using the hybrid promoter AP-1-TK construct (Fig. 3) a role for AP-1 in the mianserin-mediated regulation of the 5-HT$_2$ receptor could not be demonstrated. However, a role for the AP-1 site in receptor regulation cannot be ruled out, because the single AP-1 site linked to the basal TK promoter may not be transcriptionally equivalent to the AP-1 sites in their genuine promoter context. Probable binding sites for other transcription factors, including AP-2, NF-E1, and Polyomavirus enhancer A binding protein 3 (PEA3), were also found. We are currently investigating the possible involvement of these factors in the mianserin-mediated transcriptional down-regulation.

We have not analyzed the mechanism of 5-HT-induced down-regulation of the 5-HT$_2$ receptor; however, we can conclude, based on the exogenous receptor-reporter gene experiment (Fig. 3), that down-regulation elicited by 5-HT is distinct from that produced by mianserin. Most likely, prolonged 5-HT exposure results in a combined negative effect on the receptor, including phosphorylation, sequestration, and destabilization of receptor mRNA (3, 4).

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