5-Hydroxytryptamine$_1^A$ Receptor-Mediated Increases in Receptor Expression and Activation of Nuclear Factor-κB in Transfected Chinese Hamster Ovary Cells

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SUMMARY

The regulation in expression of human 5-hydroxytryptamine$_1^A$ (5-HT$_1^A$) receptors by agonists and antagonists was studied in a stable transfected Chinese hamster ovary cell line expressing the human 5-HT$_1^A$ receptor. Receptor density and affinity were measured with $[^{125}	ext{I}]4$-(2'-methoxyphenethyl)-1-[2'-(N-[2'-pyridinyl]-p-iodobenzamido)ethyl]piperazine ($[^{125}	ext{I}]p$-MPPI), a selective antagonist of 5-HT$_1^A$ receptors. Treatment of Chinese hamster ovary cells with serotonin or the selective agonist (±)-8-hydroxy-N,N-dipropyl-2-aminotetralin stimulated a 2.5-fold increase in receptor density. The antagonists 4-(2'-methoxyphenyl)-1-[2'-(N-[2'-pyridinyl]-p-iodobenzamido)ethyl]piperazine, (-)-(S)-pindolol, and spiperone also stimulated up-regulation of receptor expression. Agonist- and antagonist-stimulated up-regulations of receptor expression were mechanistically different. The effect of agonists was inhibited by pertussis toxin, actinomycin D, and cycloheximide. Antagonist-stimulated up-regulation was inhibited by cycloheximide, only partially inhibited by actinomycin D, and not inhibited by pertussis toxin. In the course of identifying potential pathways for coupling of the receptor to activation of transcription, we demonstrated that agonists activate the transcription regulatory factor nuclear factor-κB (NF-κB). Agonists were found to stimulate degradation of the inhibitory subunit, IκBα, and to increase the activity of a NF-κB-dependent CAT reporter gene. In contrast, the antagonist 4-(2'-methoxyphenethyl)-1-[2'-(N-[2'-pyridinyl]-p-iodobenzamido)ethyl]piperazine neither elicited degradation of IκBα nor increased reporter activity. Our data suggest that expression of 5-HT$_1^A$ receptors can be regulated by both agonists and antagonists and that the agonist but not antagonist stimulation occurs concomitantly with activation of NF-κB.

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; CHO, Chinese hamster ovary; p-MPPI, 4-(2'-methoxyphenethyl)-1-[2'-(N-[2'-pyridinyl]-p-iodobenzamido)ethyl]piperazine; TNF-α, tumor necrosis factor-α; PC, phosphatidylcholine; PLC, phospholipase C; 8-OH-DPAT, (−)-8-hydroxy-2-(3-methoxy-4-iodo-2-propenyl)amino]tetralin. DMSO, dimethylsulfoxide; MAP, mitogen-activated protein; NF-κB, nuclear factor κB; PTX, pertussis toxin; CAT, chloramphenicol acetyltransferase; HIV, human immunodeficiency virus; LTR, long terminal repeat; CMV, cytomegalovirus; IE, immediate-early; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PiPAT, [9R(+)]-trans-8-hydroxy-2-(3'-iodo-2'-propenyl)amino]tetralin.
pressed as somatodendritic autoreceptors in the dorsal and medial raphe nuclei, as well as postsynaptically in cortical and limbic structures (12, 13). The autoreceptors regulate synaptic levels of 5-HT, a neurotransmitter thought to be crucial for normal psychiatric functioning. Because particular antidepressants increase the concentration of 5-HT present in synapses, these drugs have been used in vitro to examine (indirectly) the effect of persistent increases in 5-HT on receptor expression. However, although chronic treatment with antidepressants leads to selective desensitization of 5-HT<sub>1A</sub> receptor signaling, such treatment has no effect on the density of receptor (13, 14).

Given the widespread use of transfected cells in the modeling of 5-HT<sub>1A</sub> receptor signaling, we initiated studies to examine the regulation of 5-HT<sub>1A</sub> receptors in transfected CHO cells. Prolonged treatment of cells with agonists for 5-HT<sub>1A</sub> receptors stimulated a substantial increase in receptor density. The increase was attenuated by pretreatment of the cells with PTX, as expected for a signaling pathway involving proteins sensitive to PTX (G<sub>i</sub> and/or G<sub>o</sub>). Unexpectedly, an increase in expression was also observed when cells were incubated with antagonists but was insensitive to PTX. In both instances, the increase was attenuated by cycloheximide and actinomycin, suggesting the need for mRNA and protein synthesis. In the course of searching for pathways coupling to stimulation of gene transcription, we determined that agonists for 5-HT<sub>1A</sub> receptors activate the transcription regulatory factor NF-κB. This activation of NF-κB is concomitant with up-regulation of receptors.

**Experimental Procedures**

**Materials.** [125I]p-MPPI was obtained from Dr. H. Kung (University of Pennsylvania, Philadelphia, PA) and DuPont-New England Nuclear (Boston, MA). 8-OH-DPAT, spiperone hydrochloride, and (-)-(S)-pindolol were purchased from Research Biochemicals (Natick, MA). p-MPPI was obtained from Dr. H. Kung, as well as from Research Biochemicals. PTX was purchased from Calbiochem (San Diego, CA). Polyclonal rabbit anti-IκBa/MAD-3 antibody (C-21) was obtained from Santa Cruz Biochemicals (Santa Cruz, CA).

**Cell culture.** Unless otherwise specified, the cells used in these studies were from a clonal CHO cell line transfected with the Xbal/BamHI restriction fragment of the human 5-HT<sub>1A</sub> receptor genomic clone G21 (15) subcloned into the expression vector pcDNA1/neo (InVitrogen, San Diego, CA) (16). Where specified, another clonal CHO cell line expressing human 5-HT<sub>1A</sub> receptors (provided by Dr. J. Raymond, Medical University of South Carolina, Charleston, SC) was used for comparison; this cell line is transfected with the HindIII/BamHI restriction fragment of the same human 5-HT<sub>1A</sub> receptor genomic clone G21 subcloned into the expression vector pBC12RI (15). Cells were maintained in medium containing Ham’s F12 Nutrient Mixture with L-glutamine, 10% charcoal-treated fetal bovine serum, 1% penicillin/streptomycin, and 400 μg/mL geneticin at 37° C (95% air/5% CO2). 5-HT<sub>1A</sub> receptor agonists or antagonists were added directly to the culture medium. 5-HT and 8-OH-DPAT were dissolved in H<sub>2</sub>O, whereas MPPi, spiperone, and (-)-(S)-pindolol were dissolved in DMSO.

**[125I]p-MPPI binding.** Membranes were prepared by hypotonic lysis and differential pelleting as previously described (17). Membranes were incubated in 2 ml of 50 mM Tris, pH 7.4, and 1 mM EDTA at 37° C for 30 min before centrifugation and resuspension in binding buffer (50 mM Tris, pH 7.4, containing 0.1% bovine serum albumin). Binding assays were carried out at 37° C for 40 min. Assays contained 2 μg of membrane protein and 0.05–2 nM [125I]p-MPPI in a total volume of 100 μL. Specific binding was defined with 10 μM 5-HT and was 90% at K<sub>d</sub>. Assays were terminated by the addition of 2 ml of ice-cold wash buffer (20 mM Tris, pH 7.4), and filtration was carried out using a Brandel cell harvester with glass-fiber filters (no. 32; Schleicher & Schuell, Keene, NH) presoaked with 0.5% polyethylenimine. B<sub>max</sub> and K<sub>d</sub> values were determined by Scatchard transformation of saturation binding data using unweighted linear regression analysis.

**CAT activity assay.** Cells were transfected with a plasmid constructs containing a CAT reporter gene and two copies of HIV LTR-containing tandem copies of the NF-κB binding site (X2) (provided by Drs. R. Taub and B. Stein) through calcium phosphate precipitation. The medium was replaced with serum-free medium 6 hr after transfection, and the cells were treated overnight with agonists or antagonists. CAT activity was measured according to the method described in the Promega (Madison, WI) CAT Assay kit.

**Analysis of IκBa.** Cells were serum-starved overnight and treated for 1 hr with 50 μg/mL cycloheximide to arrest protein synthesis before incubation for 2 hr with specified reagents. Cells were then washed three times with 10 ml of ice-cold saline buffer and scraped into 0.7 ml of lysis buffer (25 mM HEPES, pH 7.4, 50 mM NaF, 5 mM EDTA, 1 mM sodium orthovanadate, 100 μM phenylmethylsulfonyl fluoride, 0.1% aprotinin, and 10 μg/mL leupeptin) and passed four times through a 26-gauge needle. The lysate was centrifuged at 13,000 x g for 10 min to remove nuclei and membrane debris. Proteins were separated on 11% sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose paper, and probed with polyclonal rabbit anti-IκBα antibody at 1:100 dilution. IκBα was detected by enhanced chemiluminescence (ECL Kit, Amersham, Arlington Heights, IL).

**Results**

**Up-regulation of 5-HT<sub>1A</sub> receptors by agonists and antagonists.** Regulation of expression of 5-HT<sub>1A</sub> receptors was studied in CHO cells transfected with DNA for the human 5-HT<sub>1A</sub> receptor. Scatchard analysis revealed binding of the selective antagonist [125I]p-MPPI (19, 20) to a single class of sites. The density of receptors was 2.8 ± 0.5 pmol/mg of membrane protein, and the K<sub>d</sub> value was 0.29 ± 0.05 nm. Consistent with prior characterization of p-MPPI as an antagonist (19, 20), the K<sub>d</sub> value was not altered by guanine nucleotides or PTX. Membranes from untransfected CHO cells showed no specific binding of [125I]p-MPPI.

Incubation of CHO cells with 5-HT or the selective 5-HT<sub>1A</sub> receptor agonist 8-OH-DPAT stimulated a 2.5-fold increase in the density of receptors (Fig. 1A). Although 5-HT and 8-OH-DPAT were equally efficacious, 8-OH-DPAT was 5 times as potent (EC<sub>50</sub> = 2 μM for 5-HT versus 400 nM for 8-OH-DPAT). Interestingly, up-regulation also resulted from exposure of the cells to antagonists. The selective antagonist p-MPPI, as well as the less selective antagonists spiperone and (-)-(S)-pindolol, stimulated up-regulation of 5-HT<sub>1A</sub> receptor expression to an extent similar to that seen with agonists (Fig. 1B). The potencies of the antagonists were 2 orders of magnitude higher than that of 5-HT (EC<sub>50</sub> = 10 nM for MPPI and 50 nM for spiperone and (-)-(S)-pindolol). The time courses for agonist- and antagonist-induced receptor up-regulation were similar, with up-regulation leveling off at 12–18 hr (Fig. 2). The density of receptors after 24 hr of incubation was essentially identical to that at 18 hr (not shown). At no time was down-regulation of the receptor observed.

In most types of cells, 5-HT<sub>1A</sub> receptors couple to G proteins sensitive to PTX (G<sub>i</sub> and/or G<sub>o</sub>) (21–23). We therefore tested the effect of PTX on receptor up-regulation. Pretreat-
ment of cells with PTX for 4 hr inhibited 8-OH-DPAT-stimulated up-regulation by 70% (Fig. 3). In contrast, PTX had no effect on antagonist-induced increases in the density of receptors. The CHO cells used in these studies contain Gi but not Go (16). It therefore appears that Gi is required for a large portion of the agonist-stimulated up-regulation. Gi is not required for the actions of antagonists.

To determine whether increases in receptor density required protein synthesis, cells were treated with 5 μg/ml cycloheximide before incubation with agonists or antagonists. Cycloheximide inhibited the up-regulation stimulated by both agonists and antagonist by 75% (Fig. 4A). Increasing the concentration of cycloheximide to 10 μg/ml resulted in complete inhibition but also caused inhibition of basal levels of expression, as well as some visible cell toxicity. Because these results suggested protein synthesis was necessary for increased receptor density, we used the RNA polymerase inhibitor actinomycin D to determine whether RNA synthesis was similarly required. Incubation of the cells with actinomycin D completely inhibited agonist-induced up-regulation and caused a partial (~50%) inhibition of antagonist activity (Fig. 4B).

**Agonist activation of NF-κB.** The requirement for RNA synthesis was intriguing in the sense that 5-HT<sub>1A</sub> receptors have not been previously reported to couple to pathways modulating gene transcription, with the exception of the MAP kinases ERK1 and ERK2 (16, 24). We therefore sought to identify the relevant pathway. Translocation of active NF-κB to the nucleus is preceded by phosphorylation of the inhibitory peptide IκBa, dissociation of IκBa from NF-κB, and degradation of IκBa (25). When CHO cells expressing 5-HT<sub>1A</sub> receptors were treated for 2 hr with 8-OH-DPAT, a significant loss of IκBa was evident (Fig. 5A). p-MPPI did not stimulate IκBa degradation but instead inhibited the decrease caused by 8-OH-DPAT. Activation of NF-κB by agonists for receptors such as TNF-α receptors requires a PC-PLC, which is selectively inhibited by D609 (tricyclodecan-9-
Preparation of CHO cells with 50 μg/ml D609 prevented 8-OH-DPAT-stimulated degradation of IκBα (Fig. 5B).

To directly examine stimulation of NF-κB by agonists, we used a NF-κB reporter gene. CHO cells were transiently transfected with a construct containing two copies of the HIV LTR with tandem copies of NF-κB binding sites linked to a CAT reporter (18). Treatment of cells for 18 hr with 8-OH-DPAT stimulated a 75% increase in measured CAT activity (Fig. 6). The vehicle for 8-OH-DPAT was H2O, which had no effect on activity. DMSO, the vehicle for p-MPPI, caused a small increase in activity. p-MPPI had no further effect (Fig. 6) and inhibited the stimulation by DPAT (not shown).

**Lack of up-regulation in a CHO cell line transfected with a different expression vector.** Because our findings suggested that up-regulation of receptor was a result of increased transcription, we examined the role of the promoter. All of the experiments described above used a CHO cell line that had been transfected with the XbaI/BamHI restriction fragment of the human 5-HT1A receptor genomic clone G21 subcloned into the expression vector pcDNA1. This vector contains the CMV IE enhancer/promoter, which has been shown to contain binding sites for and to be activated by NF-κB (27, 28). For comparison, we also examined a different CHO cell line that had been transfected with the G21 restriction fragment subcloned into the vector pBC12BI (15). pBC12BI does not contain a CMV promoter, but instead has a Rous sarcoma virus LTR, which functions as the promoter. The RSV promoter does not contain consensus NF-κB binding sites, nor has it been reported to be activated by NF-κB. The pBC12BI-transfected CHO cells expressed 5-HT1A receptors at 1.8 ± 0.3 pmol/mg of membrane protein, a density similar to that expressed by the pcDNA1-transfected cells. Significantly, overnight incubation with 8-OH-DPAT did not stimulate a change in expression of receptors in pBC12BI-transfected cells.

**Discussion**

In our study, we found that both agonists and antagonists stimulated increased expression of human 5-HT1A receptors in cells transfected with a vector containing a CMV promoter. This stimulation required almost complete occupancy of receptors. The EC50 values for p-MPPI and 8-OH-DPAT were 10 and 400 nM, respectively, which is in contrast to KD values of 0.29 and 2 nM. We also found that up-regulation of receptor requires continuous incubation with ligands. If cells were treated for several hours with 8-OH-DPAT and binding was measured several hours after the agonist was removed, there was no increase in receptor expression (not shown). This suggests that the process of up-regulation requires continual occupancy of a high percentage of the receptors. Because 5-HT1A receptors are known to desensitize, one could postu-
late that high ligand concentrations are necessary to maintain prolonged activation of a required cellular pathway.

Although both agonists and antagonists caused increased expression of receptors, the two types of ligands used different pathways. The inhibition by PTX of the agonist-stimulated up-regulation was consistent with a mechanism involving Gα or Gβγ. PTX also attenuates the inhibition of adenyl cyclase (not shown) and activation of MAP kinase (16) triggered by 8-OH-DPAT. In contrast, PTX had no effect on antagonist-stimulated up-regulation. The sensitivity to PTX suggests that the pathway activated by agonists, but not antagonists, is mediated by Gα. It is interesting to note that other receptors coupled to Gα have been shown to up-regulate in response to treatment with agonists. It was previously demonstrated in transfected human embryonic kidney 293 cells that both agonists and antagonists stimulate increased expression of D2L dopamine receptors (7). Significantly, in transfected CHO cells, it has also been reported that agonists stimulate increases in the density of D2L receptors (9).

It is interesting that both agonists and antagonists stimulate increased expression of receptor. In vivo, β-adrenergic receptor agonists stimulate down-regulation, whereas antagonists stimulate up-regulation. The antagonist effect is thought to be a result of prevention of down-regulation by an available agonist. Our results are similar to those seen for D2L dopamine receptors, in which both agonists and antagonists individually stimulate increased expression in transfected cells (7). We have so far been unsuccessful in identifying specific second messenger systems activated by antagonists. In contrast to agonists, p-MPPI does not inhibit adenyl cyclase (not shown) or activate MAP kinases (16). p-MPPI also does not activate NF-κB. In transfected HeLa cells, 5-HT1A receptors couple to phosphoinositide hydrolysis (29); however, we found no activation by agonists or antagonists of phosphoinositide hydrolysis in CHO cells (not shown). We are continuing to search for cellular pathways that might be activated by antagonists. It is possible that a tyrosine kinase or another enzyme involved in signaling associates with 5-HT1A receptors and can be stimulated by antagonists independent of G proteins. Although we have no evidence that p-MPPI is anything but a neutral antagonist, induction of a change in the conformation of receptor on binding of p-MPPI (or (-)-(S)-pindolol and spiperone) may result in activity of a nature similar to inverse or partial agonism.

Our results in CHO cells contrast with those of Harrington et al. (30), who reported a large (almost 80%) decrease in Bmax of [3H]8-OH-DPAT binding in transfected HeLa cells 10 min after treatment with 8-OH-DPAT. We found no loss of receptors at 10 min or any other time point. Our studies measured receptor binding with the antagonist [125I]l-MPPI. It is possible that the use of [3H]8-OH-DPAT as a radiolabeled ligand resulted in observed differences; however, we obtained identical results when the selective agonist [125I]8-OH-PIPAT (31) was used to measure receptor density (not shown). Because 8-OH-PIPAT and 8-OH-DPAT are both agonists and are chemically similar, they would be expected to provide similar results. Alternatively, cellular differences in G protein coupling and second messenger systems may have been responsible for the differences. Agonists at 5-HT1A receptors inhibit adenyl cyclase and activate phosphoinosi-
tide hydrolysis in HeLa cells (29, 30) but only inhibit adenyl cyclase in CHO cells.

Our finding that 8-OH-DPAT stimulates NF-κB represents the first report of 5-HT1A receptor coupling to a specific transcription regulatory factor. Activation of NF-κB has been best characterized for TNF-α receptors (26). NF-κB is normally retained in the cytosol in an inactive form, bound to IκBα. Dissociation of IκBα occurs concomitantly with phosphorylation and ubiquitination (32, 33). Although the pathways that stimulate these events have not been completely defined, components of the MAP kinase signaling system have been implicated. NF-κB activity is increased by transfection of cells with constitutively active forms of ras, raf, or mitogen-activated protein kinase kinase (34, 35). Conversely, dominant negative forms of ras and mitogen-activated protein kinase kinase inhibit stimulation by agonists such as TNF-α. There is evidence that ras stimulates a PC-PLC (36) and that PC-PLC is required for activation of NF-κB, presumably through a process involving sphingomyelin breakdown (26). Our finding that 8-OH-DPAT stimulates MAP kinase (16) and that the degradation of IκBα is inhibited by D609 is consistent with a similar mechanism. The connection between G protein-coupled receptors and NF-κB may indeed be extensive; Kravchenko et al. (37) recently demonstrated activation of NF-κB by platelet-activating factor.

Our studies lead to the hypothesis that the two events examined (up-regulation and NF-κB activation) may be connected. The expression vector used in our studies, pcDNA1-neo, contains a CMV IE enhancer/promoter commonly used to drive expression of transfected receptor cDNA. This promoter contains binding sites for a number of transcription factors, including ATF/CREB, MEF1, SPI, and NF1, as well as NF-κB (27, 28, 38–40). Of note, the activity of the CMV IE enhancer/promoter has been previously reported to be enhanced by NF-κB (27, 28). It is possible that 8-OH-DPAT and 5-HT stimulate receptor expression by activating NF-κB, thereby increasing the activity of this promoter. In support of this hypothesis, we found no up-regulation of 5-HT1A receptors when CHO cells were transfected with the pBC12BI vector, which contains a Rous sarcoma virus LTR as a promoter instead of a CMV promoter. Significantly, the consensus NF-κB binding sequences CGGGACTTTC and GGG-GATTTC (27) are not present in the Rous sarcoma virus LTR, and NF-κB has not been reported to enhance the activity of the promoter. The use of transfected cells as a model for up-regulation must therefore be viewed cautiously. Of the reports dealing with up-regulation in transfected cells, most if not all used plasmids containing the CMV IE enhancer/promoter or other viral promoters that might be similarly regulated.

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