Regulation of mRNA Encoding 5-HT<sub>2A</sub> Receptors in P11 Cells Through a Post-transcriptional Mechanism Requiring Activation of Protein Kinase C

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Exposure of P11 cells to serotonin (5-HT) resulted in a transient increase in levels of 5-HT<sub>2A</sub> receptor mRNA. Exposure to 5-HT for as short a time as 1 min was sufficient to trigger a delayed increase in receptor mRNA. 5-HT-induced increases in receptor mRNA levels were not antagonized by the protein synthesis inhibitor cycloheximide. The increase in receptor mRNA levels was accompanied by a transient increase in the half-life of receptor mRNA; the rate of transcription of receptor mRNA was unchanged. Submaximal stimulation of phosphoinoside hydrolysis by partial agonists or 6-fluorom nepinophine, an α<sub>1</sub>-adrenergic receptor agonist, also increased receptor mRNA levels. Exposure to phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, mimicked these effects, whereas the protein kinase C inhibitor bisindolylmaleimide antagonized the effects of both 5-HT and PMA. When agonist-promoted increases in receptor mRNA were prevented, the rate of agonist-induced down-regulation was accelerated. These data suggest that levels of 5-HT<sub>2A</sub> receptor mRNA are regulated by phospholipase C-coupled receptors via a protein kinase C-dependent, post-transcriptional mechanism and indicate that agonist-promoted increases in levels of 5-HT<sub>2A</sub> receptor mRNA modulate receptor expression.

The neurotransmitter 5-hydroxytryptamine (5-HT<sup>+</sup>) interacts with multiple subtypes of receptors (1, 2) to elicit diverse effects. One of the first 5-HT receptor subtypes to be cloned was the 5-HT<sub>1A</sub> receptor (3), formerly called the 5-HT<sub>2</sub> receptor (1). This receptor is a member of the G protein-linked receptor superfamily and is coupled to the stimulation of PI hydrolysis. Numerous studies of the regulation of this receptor in the central nervous system have been carried out. Blackburn et al. (4) reported that administration of trifluromethylphenylpiperazine, a 5-HT<sub>1A</sub> receptor agonist, resulted in a decrease in the density of 5-HT<sub>1A</sub> receptors in rat frontal cortex. Similar results have been observed after administration of other full or partial agonists, including quipazine (5), LSD, DOB, and DOI (6). Paradoxically, antagonists of 5-HT<sub>2A</sub> receptors (e.g. mianserin, ketanserin, metergoline, methysergide, and cyproheptadine) have also been observed to cause down-regulation of receptors in vivo (7, 8). However, problems of residual drug in binding assays and possible indirect effects of these agents have complicated interpretation of results of studies carried out in vivo using antagonists.

P11 cells, an immortalized cell line derived from transplantable rat pituitary tumor 731sa (9), have been used as a model system to study regulation of 5-HT<sub>2A</sub> receptors. 5-HT<sub>2A</sub> receptors in this cell line are rapidly desensitized following exposure to agonists (10), an effect which appears to be mediated in part by a decrease in the density of receptors (10, 11). Agonists and partial agonists, but not antagonists, were found to down-regulate 5-HT<sub>2A</sub> receptors in P11 cells (11). These findings suggested that agonist and antagonist-promoted receptor down-regulation in vivo may proceed through distinct mechanisms, with the effects of agonists likely to be the result of interactions with the receptors. However, mechanisms responsible for agonist-induced down-regulation of receptors have not been identified.

Levels of neurotransmitter receptors can be modulated through regulation of levels of mRNA encoding the receptors. Transcriptional regulation has been described for several G protein-linked receptors, including β-adrenergic (12), α-adrenergic (13), dopamine (14), muscarinic acetylcholine (15), and thyrotropin-releasing hormone (16) receptors. The most widely studied of these receptors is the β<sub>2</sub>-adrenergic receptor, for which a biphasic pattern of receptor mRNA regulation has been observed. Acute exposure to agonists or agents that elevate cAMP levels results in a 3-4-fold increase in levels of β<sub>2</sub>-adrenergic receptor mRNA (17). The increase has been shown to result from an increase in the rate of transcription of the β<sub>2</sub>-adrenergic receptor gene, an effect mediated by a cAMP-response element in the proximal promoter region of the gene (12, 17). In contrast, chronic exposure to agonists results in a 50% decrease in β<sub>2</sub>-adrenergic receptor mRNA levels (17, 18). This decrease, which is due to a decrease in receptor mRNA stability (19), is thought to contribute to receptor down-regulation observed after prolonged exposure to agonists (12, 20).

Several studies of the regulation of 5-HT<sub>2A</sub> receptor mRNA expression have been carried out, but inconsistent results have been obtained. Prolonged administration of antagonists to rats has been squelched to cause an increase (21) or to elicit no change (22) in levels of 5-HT<sub>2A</sub> receptor mRNA. Results of studies using primary cells in culture have shown that exposure to agonists results in an increase in levels of 5-HT<sub>2A</sub> receptor mRNA (23, 24), whereas exposure to antagonists caused no change in levels of receptor mRNA (25). An extensive time course of changes in mRNA levels in response to drug treat-
ment was not measured in these studies. In addition, intracellular mediators of the changes in levels of receptor mRNA were not investigated, making it difficult to determine whether the observed inconsistencies were due to cell type-specific mechanisms or were the result of artifacts of primary cell culture systems, which often contain multiple populations of cells. The objectives of the current study were to investigate transcriptional regulation of 5-HT<sub>2A</sub> receptors and to identify mechanisms which regulate expression of 5-HT<sub>2A</sub> receptor mRNA. An antisense riboprobe specific for 5-HT<sub>2A</sub> receptor mRNA was constructed and used in ribonuclease protection assays to quantify levels of 5-HT<sub>2A</sub> receptor mRNA in P11 cells. Mechanisms responsible for regulating levels of 5-HT<sub>2A</sub> receptor mRNA were explored and intracellular messengers participating in these events were investigated.

**EXPERIMENTAL PROCEDURES**

**Materials**—[methyl-<sup>3</sup>H]Inositol (20 Ci/mmol), [alpha-<sup>32</sup>P]CTP (800 Ci/mmol), [3H]-LSD (2200 Ci/mmol), and [alpha-<sup>32</sup>P]UTP (9000 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Bisindolylmaleimide (GF 109203X) was purchased from Calbiochem. Components of media for cell culture were purchased from Life Technologies, Inc., except fetal bovine serum, which was obtained from Hyclone (Logan, UT). Remaining drugs and chemicals were purchased from Sigma, except where otherwise indicated.

**Cell Culture**—P11 cells (9) were grown in monolayer culture at 37 °C in high glucose Dulbecco's modified Eagle medium (supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml 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<sub>2</sub>. Cells were detached with 0.25% trypsin and 1 mM EDTA at 37 °C and resuspended in 5 ml of 3 M LiCl. Samples were centrifuged at 16,000 × g, washed with 80% ethanol, and vacuum-dried. Samples were dissolved in water and heated to 90 °C for 5 min and then with proteinase K, and extracted with phenol/chloroform/isoamyl alcohol (25:24:1, pH 5.2), samples were precipitated on dry ice for 30 min with 2 volumes of ethanol in the presence of 30 μg of yeast tRNA, which served as a carrier to facilitate precipitation. Samples were centrifuged, washed with 70% ethanol, and vacuum-dried. Samples were then dissolved in formamide loading buffer and size fractionated on a 7.8% polyacrylamide gel. Dried gels were apposed to phosphor storage screens for 5–20 h and then scanned and digitized using a Molecular Dynamics model 4005 PhosphorImager. The signal corresponding to the 5-HT<sub>2A</sub> receptor riboprobe fragment was normalized to that of the cyclophilin signal from the same sample to control for experimental variability.

**Radioligand Binding**—Binding assays using [methyl-<sup>3</sup>H]LSD to label 5-HT<sub>2A</sub> receptors in membrane homogenates obtained from P11 cells were carried out as described previously (11). Nonspecific binding was defined using 1 μM ketanserin.

**Measurement of PI Hydrolysis**—Inositol phosphates were collected after addition of LiCl and analyzed as described previously (11).

**Isolation of RNA**—P11 cells were detached from plates with trypsin, centrifuged for 10 min at 500 × g, and resuspended in phosphatebuffered saline. Cells were frozen and stored at −80 °C until processed. RNA was isolated from frozen P11 cells and frozen tissue samples by LiCl precipitation essentially as described by Cathala et al. (26). Briefly, frozen cells from one 15-cm plate or 0.75 g of tissue were homogenized using a Brinkmann polytron (speed 7 for 1.5 min) in 5 ml of a solution containing 5 μg guanidinium isothiocyanate, 50 mM Tris, pH 7.5, and 0.5 μg/mL LiCl was then added to a final volume of 40 ml. After precipitation overnight at 4 °C, samples were centrifuged at 16,000 × g for 90 min, and the resulting pellets were resuspended in 5 ml of 3 M LiCl. Samples were centrifuged at 16,000 × g for 1 h and the resulting pellets resuspended in 2 ml of buffer containing 0.1% SDS, 1 mM EDTA, pH 8.0, and 0.5 μg/mL dithiothreitol. 5-HT<sub>2A</sub> receptor binding was determined by addition of ω-conotoxin GVIA (25:24:1, pH 5.2) and with chloroform/isooamyl alcohol (24:1). Samples were precipitated overnight at −20 °C. RNA pellets were collected by centrifugation for 30 min at 16,000 × g, washed with 80% ethanol, and vacuum-dried. Samples were stored at −80 °C in RNase-free water containing 80 mM sodium acetate and 70% ethanol.

**Construction of Recombinant Plasmids for Synthesis of Riboprobes**—Using rat 5-HT<sub>2A</sub> receptor cDNA as the template, a portion of the third intracellular loop corresponding to nucleotides 1380–1577 of the published sequence (3) was amplified by the polymerase chain reaction using forward (sense) primer, 5′-TCA CTT CCT CAA AAA GAA TAC TCC ACC TTG GTG-3′ and reverse (antisense) primer, 5′-TAG ACT ACT CAA GCT TGC ACG CCT TTT TGT CAT CGT-3′. Amplified cDNA was ligated into the EcoRI and HindIII sites of pGEMTZ-6+ (Promega Corp., Madison, WI) to generate a recombinant plasmid (5-HT<sub>2A</sub>-<sup>32</sup>P)2B. This construct was used to transform E. coli HB101 cells. Transformed cells were grown on LB agar plates, single colonies were expanded, and plasmid DNA was isolated using alkaline lysis followed by centrifugation through pZ523 columns (5 Prime-3 Prime, Inc., Boulder, CO). Sequencing of cloned cDNA inserts was carried out using a Sequenase Version 2.0 DNA sequencing kit (United States Biochemical, Cleveland, OH). Preparation of cyclophilin clone pB15, containing a 700-base pair sequence of the coding region of a cyclophilin, has been described elsewhere (27).

**Synthesis of Riboprobes**—Plasmids containing a CDNA fragment encoding a portion of the third intracellular loop of the 5-HT<sub>2A</sub> receptor (5-HT<sub>2A</sub>-<sup>32</sup>P)2B and a 700-base pair CDNA fragment encoding cyclophilin were linearized with EcoRI and HindIII, respectively. SF9 phage RNA polymerase (Promega Corp.) was used to synthesize 5-HT<sub>2A</sub>-<sup>32</sup>P and cyclophilin antisense RNA transcripts of 228 and 728 bases, respectively. Conditions used were: 2 μg of template, 1 μl each of 10 mM ATP, GTP, and UTP, 2 μl of 0.1 M DTT, 40 units of recombinant RNase inhibitor (rRNasin; Promega Corp.), 40 units of RNA polymerase, 5 μl of transcription buffer (Promega Corp.), 90 μl of [alpha-<sup>32</sup>P]CTP (6,000 Ci/mmol), and 0.5 μl of 0.1 μM CTP (5-HT<sub>2A</sub> riboprobe) or 2 μl of 10 mM CTP (cyclophilin riboprobe) in a total volume of 25 μl. Reactions were incubated for 90 min at 38 °C, after which 2 units of RNase-free DNase (Promega Corp.) were added to digest the template. Riboprobes were separated from unincorporated nucleotides by centrifugation through a 5-μl 20–80% sucrose gradient. Partial chloroacetic acid precipitation and liquid scintillation spectroscopy were used to measure the amount of label incorporated into the probe. The integrity of riboprobes was assessed by size fractionation of an aliquot of each riboprobe on a polyacrylamide gel followed by autoradiography.

**Ribonuclease Protection Assay**—An aliquot of total RNA (10 μg) from each sample was placed into a 1.5-ml microfuge tube and vacuum-dried. Hybridization buffer (40 μl of buffer consisting of 80% deionized formamide with 400 μM NaCl, 40 μM Tris, pH 6.4, and 2 mM EDTA, pH 8.0) plus 500,000 counts of the 5-HT<sub>2A</sub> riboprobe and 50,000 counts of the cyclophilin riboprobe were added to each tube, and samples were allowed to hybridize at 45 °C for 14–18 h. After hybridization, the mixture was digested with 2 μg of RNase A and 1900 units of RNase T1 (Promega Corp.) for 90 min at room temperature in buffer containing 300 mM NaCl, 20 mM Tris, pH 7.4, and 5 mM EDTA, pH 8.0. SDS (23 μl of 0.5%) containing 60 μg of proteinase K (Boehringer Mannheim) was added to each tube, followed by incubation at 37 °C for 20 min. Following extraction with phenol/chloroform/isooamyl alcohol (25:24:1, pH 5.2), samples were precipitated on dry ice for 30 min with 2 volumes of ethanol in the presence of 30 μg of yeast tRNA, which served as a carrier to facilitate precipitation. Samples were centrifuged, washed with 70% ethanol, and vacuum-dried. Samples were then dissolved in formamide loading buffer and size fractionated on a 7.8% polyacrylamide gel. Dried gels were apposed to phosphor storage screens for 5–20 h and then scanned and digitized using a Molecular Dynamics model 4005 PhosphorImager. The signal corresponding to the 5-HT<sub>2A</sub> receptor riboprobe fragment was normalized to that of the cyclophilin signal from the same sample to control for experimental variability.

**Nuclear Run-on Assays**—Cells from one 10-cm plate (approximately 4 × 10⁷ cells) were used for isolation of nuclei according to the alternate protocol described by Greenberg and Bender (25). Briefly, cells were washed with 70% ethanol, and vacuum-dried. Samples were then dissolved in formamide loading buffer and size fractionated on a 7.8% polyacrylamide gel. Dried gels were apposed to phosphor storage screens for 5–20 h and then scanned and digitized using a Molecular Dynamics model 4005 PhosphorImager. The signal corresponding to the 5-HT<sub>2A</sub> receptor riboprobe fragment was normalized to that of the cyclophilin signal from the same sample to control for experimental variability.
receptor (in pGEM7zf(+), see Construction of Recombinant Plasmids for Synthesis of Riboprobe), or vector DNA immobilized on nitrocellulose membranes. Hybridizations using equivalent amounts of labeled RNA were carried out at 42 °C for 60 h after prehybridization for 6 h in hybridization solution lacking radiolabeled RNA. The hybridization solution contained (final concentrations): 6 × SSC (0.9 × NaCl, 0.1 × citric acid), 50% formamide, 100 μg/ml of sheared salmon sperm DNA, 5 × Denhardt’s Solution, and 0.5% SDS. Membrane strips were washed in 2 × SSC, 0.5% SDS for 5 min at room temperature, 2 × SSC, 0.1% SDS for 15 min at room temperature, 0.1 × SSC, 0.5% SDS for 30 min at 37 °C, and in 0.1 × SSC, 0.5% SDS for 30 min at 52 °C. Autoradiograms were generated by exposing membrane strips to film and intensifying screens for 5 days at −80 °C. For quantitation, dried membrane strips were apposed to phosphor storage screens for 20 h and then scanned and digitized using a Molecular Dynamics model 4005 PhosphorImager.

Statistics—All experimental values are reported as means ± standard errors. Individual experiments included three or more independent determinations and were carried out on two or more separate occasions. Individual determinations, either within a given experiment or from several independent experiments, are listed as number of determinations or “n =.” For binding experiments, data are shown as means ± standard errors of three determinations each performed in triplicate. Comparisons of the data were made with a one-way analysis of variance (ANOVA) using StatView software for the Macintosh. The degree of certainty of statistically significant differences among data within a given experiment or between replicate experiments is indicated by a p value which is reported in the figure legends and in the text, respectively.

RESULTS

Ribonuclease Protection Assay for 5-HT2A Receptor mRNA—Based on the reported sequence of the 5-HT2A receptor, a 32P-labeled riboprobe which specifically recognized mRNA encoding the third intracellular loop of the 5-HT2A receptor was generated (see “Experimental Procedures”). Hybridization of this riboprobe to total RNA isolated from P11 cells, followed by digestion with single strand-specific ribonucleases and denaturing polyacrylamide gel electrophoresis, yielded a protected riboprobe fragment of 183 bases (Fig. 1). Cohybridization with a 32P-labeled cyclophilin riboprobe yielded a second protected fragment of 700 bases. When compared to the amount of total RNA added to each tube (assessed by absorbance at 260 nm), levels of cyclophilin mRNA were found to be unaffected by exposure to a variety of drugs, including those used in this study. The 5-HT2A receptor signal was normalized to that of the cyclophilin signal appearing in the same lane.

Full-length sense 5-HT2A receptor RNA synthesized in vitro and hybridized to the 5-HT2A receptor-specific riboprobe identified the position of the protected riboprobe fragment and served as a positive control (Fig. 1, lane 2). A band in an identical position was seen when the riboprobe was hybridized to total RNA from rat forebrain, a tissue rich in 5-HT2A receptors (Fig. 1, lane 1). (Upper band in lane 1 represents protected cyclophilin riboprobe fragment.) Hybridization to non-homologous RNA (Fig. 1, lane 3) and samples containing no RNA (Fig. 1, lane 4) served as negative controls.

Effects of 5-HT on Levels of 5-HT2A Receptor mRNA—Exposure to 5-HT resulted in an increase in 5-HT2A receptor mRNA levels (Fig. 1, lanes 7–9 and 10–12). The increase in mRNA after exposure to 5-HT was time dependent, with significant increases occurring within 30 min (Fig. 2). Levels of receptor mRNA were maximally increased in cells treated with 10 μM 5-HT for 90 min (Fig. 2). In three experiments, mRNA levels in cells treated for 90 min were increased to 177–226% of control levels (195 ± 4%, n = 11, p < 0.001). The increase in levels of receptor mRNA was dose dependent, with an EC50 of approximately 400 nM (data not shown). Levels of receptor mRNA were elevated only transiently and returned to control levels within 8–16 h (Fig. 2, inset). Continued exposure to 5-HT for up to 48 h caused no further change in the levels of receptor mRNA compared to control values. For this prolonged incubation, 5-HT was added every 12 h to ensure that a sufficient concentration of 5-HT was present to fully occupy the receptors during the entire course of the experiment. This redosing schedule was based on the half-life of serotonin in cell culture, which was determined using high pressure liquid chromatography to be 13.6 h (data not shown).
Cycloheximide was added and the incubation allowed to continue for a total of 90 min. Ketanserin prevented further activation of 5-HT$_{2A}$ receptors without the need to include a wash step to remove 5-HT. Using this experimental paradigm, exposure to 5-HT for 1 min was observed to result in a 38% increase in levels of 5-HT$_{2A}$ receptor mRNA when measured 85 min after ketanserin was added (Fig. 3). In two independent experiments, a 1-min exposure caused a 25–38% increase in receptor mRNA levels (32 ± 5%, $n = 6$, $p < 0.005$). This effect was time dependent; a 5-min exposure to 5-HT caused a 52% increase in receptor mRNA when measured 85 min after addition of ketanserin, and a 20-min exposure to 5-HT caused a 68% increase when measured 70 min later (Fig. 3). In two separate experiments, a 5- and 20-min exposure to 5-HT resulted in delayed increases in receptor mRNA of 28–53% (38 ± 6%, $n = 6$, $p < 0.005$) and 63–73% (69 ± 12%, $n = 6$, $p < 0.005$), respectively. Ketanserin alone had no effect on receptor mRNA levels but completely antagonized the 5-HT-mediated increase (Fig. 3, inset).

The design of this experiment required that ketanserin immediately block further activation of 5-HT$_{2A}$ receptors by 5-HT. The ability of ketanserin to abolish the agonist activity of 5-HT was tested by adding ketanserin to cells that had been exposed to 10 µM 5-HT for 5 min. Levels of inositol phosphates measured 1–25 min after the addition of ketanserin were the same as those seen after exposure to 5-HT for 5 min (data not shown). Because 5-HT causes a time-dependent increase in inositol phosphate levels (9), this indicated that ketanserin promptly antagonized the agonist activity of 5-HT.

**Effects of Cycloheximide on Levels of 5-HT$_{2A}$ Receptor mRNA**—To determine whether the increase in 5-HT$_{2A}$ receptor mRNA levels after exposure to 5-HT required de novo protein synthesis, cells were treated with the inhibitor of protein synthesis cycloheximide (2.5 µg/ml) for 30 min prior to exposure to 5-HT for 60 min. This dose of cycloheximide inhibited protein synthesis by 96.9 ± 0.3% in control cells and by 97.3 ± 0.1% in 5-HT-treated cells as measured by incorporation of [H]leucine ($n = 4$). Cycloheximide itself caused a small increase in levels of receptor mRNA but did not prevent 5-HT from elevating receptor mRNA levels (Fig. 4); levels of receptor mRNA in cells exposed to 5-HT for 1 h were increased by 158–170% of control levels (165 ± 3%, $n = 4$, $p < 0.001$) in vehicle-treated cells and 147–157% (153 ± 2%, $n = 4$, $p < 0.001$) in cycloheximide-treated cells.

**Effects of 5-HT on the Rate of Transcription of the 5-HT$_{2A}$ Receptor Gene**—To determine whether changes in the rate of transcription were responsible for 5-HT-induced changes in 5-HT$_{2A}$ receptor mRNA levels, nuclear run-on assays were carried out after harvesting nuclei from cells treated with 5-HT for 45 min or 2.5 h. (Receptor mRNA levels are increasing (45 min) or decreasing (2.5 h) at these times.) Newly elongated transcripts were hybridized to membranes containing immobilized full-length 5-HT$_{2A}$ receptor cDNA or cDNA encoding the third intracellular loop of the 5-HT$_{2A}$ receptor (Fig. 5A). The rate of 5-HT$_{2A}$ receptor gene transcription was found to be unchanged at both times as measured using either cDNA as template (Fig. 5B). Similarly, no significant change in the relative rate of transcription was observed after a 5- (0.90 ± 0.08, versus control; $n = 4$) or 10- (0.96 ± 0.13) min incubation with 5-HT.

**Effects of 5-HT on the Stability of 5-HT$_{2A}$ Receptor mRNA**—Because exposure to 5-HT did not alter the rate of 5-HT$_{2A}$ receptor gene transcription, turnover of receptor mRNA was studied in 5-HT-treated cells by measuring receptor mRNA levels at various times after inhibiting transcription with actinomycin D. Receptor mRNA turnover was examined after incubating cells with 5-HT for 45 min or 2.5 h, when levels of receptor mRNA were approximately equal (Fig. 2). (Receptor mRNA levels are increasing (45 min) or decreasing (2.5 h) at these times.) Receptor mRNA in cells treated with 5-HT for 45 min decreased more slowly after the addition of actinomycin D than in vehicle-treated cells (Fig. 6). A semi-logarithmic plot of the data in this experiment revealed that 5-HT increased the half-life of 5-HT$_{2A}$ receptor mRNA by approximately 75% (Fig. 6, inset A). In three independent experiments mRNA stability was increased from 75–12% (95 ± 11%, $n = 9$, $p < 0.001$). The increase in receptor mRNA half-life was transient; following a 2.5-h exposure to 5-HT, the half-life of receptor mRNA returned to control values (Fig. 6, inset B). In three separate experiments ($n = 9$), no significant difference ($p > 0.2$) in receptor mRNA half-life was observed in cells treated with 5-HT for 2.5 h ($t_{1/2} = 71–98$ min; average $t_{1/2} = 82 ± 8$ min) compared to control values (65–76 min; 70 ± 3 min). Incubation with actinomycin D for up to 180 min did not decrease levels of cyclophilin mRNA, indicating that turnover of cyclophilin
Fig. 5. Relative rate of transcription of the 5-HT2A receptor gene following exposure to 5-HT as assessed by nuclear run-on assays. A, P11 cells grown for 6 days in 10-cm dishes were exposed to 10 μM 5-HT for 45 min or 2.5 h. Cells were harvested, nuclei were isolated, and nuclear run-on assays were carried out in the presence of 250 μCi of [α-32P]UTP as described under "Experimental Procedures." Equal amounts of newly elongated RNA were hybridized to linearized 5-HT2A receptor cDNA (1), cDNA encoding the third intracellular loop of the 5-HT2A receptor (3), or to plasmid lacking receptor cDNA inserts (pVL1393 (2), or pGEM7zf(+)) (4), respectively) as controls. Blots were exposed to film and intensifying screens for 5 days at –80 °C. B, phosphor storage screens exposed to the blots shown in A for 24 h were scanned and digitized on a Molecular Dynamics PhosphorImager. Background hybridization to plasmids not containing cDNA inserts was subtracted to account for nonspecific hybridization. Data shown are expressed relative to control levels and represent means ± standard errors of duplicate determinations. Similar results have been obtained in two separate experiments.

Fig. 6. Changes in the stability of 5-HT2A receptor mRNA after exposure to 5-HT. P11 cells were treated with 10 μM 5-HT or vehicle (H2O) for 45 min or 2.5 h before the addition of actinomycin D (5 μg/ml). Cells were harvested at various times after the addition of actinomycin D, and receptor mRNA levels were measured using a ribonuclease protection assay. Shown in the main panel are results obtained from cells that were treated with 5-HT for 45 min. Inset, semi-logarithmic plots of 5-HT2A receptor mRNA decay following inhibition of transcription with actinomycin D in cells treated with 5-HT for 45 min (A) or 2.5 h (B). Receptor mRNA half-lives (t1/2) were calculated by linear regression of ln (M/M0) versus time, where M0 = receptor mRNA levels at given time (t), and M = receptor mRNA levels at time zero. Data are shown as means ± standard errors (n = 3). This experiment has been repeated twice with similar results. *, p < 0.05 versus vehicle; **, p < 0.01; ***, p < 0.001.

mRNA is slower than that of 5-HT2A receptor mRNA.

Effects of LSD, DOI, and FENE on Levels of 5-HT2A Receptor mRNA—Compared to 5-HT, LSD and DOI were partial agonists at stimulating PI hydrolysis (Fig. 7A) and increasing levels of 5-HT2A receptor mRNA (Fig. 7B). Incubation of P11 cells with 1 μM LSD or 100 nm DOI for 90 min resulted in 38 ± 10% (25–58%, n = 3) and 33 ± 3% (28–38%, n = 3) increases, respectively, in receptor mRNA levels. Stimulation of α1-adrenergic receptors with FENE, an α1-adrenergic receptor agonist which causes an increase in PI turnover similar in magnitude to that seen with LSD and DOI (Fig. 7A), elicited a 21 ± 1% (20–22%, n = 3) increase in the levels of 5-HT2A receptor mRNA (Fig. 7B). The effect of FENE was not a consequence of a nonspecific interaction with 5-HT2A receptors because homologous desensitization of 5-HT2A receptors on P11 cells does not reduce the ability of FENE to stimulate PI hydrolysis (10).

Effects of Modulators of Protein Kinase C on Levels of 5-HT2A Receptor mRNA—Treatment of P11 cells with 100 nm PMA for 105 min resulted in a 2-fold increase in 5-HT2A receptor mRNA levels (Fig. 8), an increase that was similar in magnitude to that observed after continuous exposure to 10 μM 5-HT for 90 min (Fig. 8). Coincubation with 5-HT for 90 min did not lead to an additional increase in receptor mRNA, indicating that levels of mRNA were maximally increased by either treatment and suggesting that the effects of the drugs operate through common mechanisms. Exposure of cells to the inactive phorbol ester 4a-phorbol 12,13-didecanoate did not alter receptor mRNA levels and did not interfere with the ability of 5-HT to increase receptor mRNA levels (Fig. 8). Bisindolylmaleimide (GF 109203X), a potent and selective inhibitor of protein kinase C (29), had no effect on 5-HT2A receptor mRNA levels, but fully antagonized the effects of PMA (Fig. 8). Bisindolylmaleimide also completely prevented 5-HT from increasing receptor mRNA levels (Fig. 8).

Rate of 5-HT2A Receptor Down-regulation in the Presence or Absence of Increases in Levels of Receptor mRNA—If agonist-induced increases in levels of 5-HT2A receptor mRNA are translated into functional protein, the rate of receptor down-regulation in the absence of agonist-promoted increases in receptor mRNA would be expected to be faster than in cells in which the increase in receptor mRNA occurred. Actinomycin D, an inhibitor of transcription, was used to prevent agonist-stimulated increases in levels of receptor mRNA. Exposure of P11 cells to actinomycin D prevented 5-HT from increasing receptor mRNA levels and resulted in a marked decrease in levels of 5-HT2A receptor mRNA (Table I) without affecting the density of receptors (after a 5-h incubation, receptor density was 99.5 ± 2.9% of control levels; n = 3). Levels of receptor mRNA in cells treated with actinomycin D or actinomycin D and 5-HT were similar, approximately 30% of control levels, whereas levels of receptor mRNA in cells treated with 5-HT alone were increased by approximately 75% over control levels. In cells treated with actinomycin D and 5-HT, the rate of receptor down-regulation was
Acute Exposure to 5-HT leads to an increase in receptor mRNA levels (Fig. 3), the effects on receptor density of brief receptor stimulation were studied. Receptor down-regulation activated by 5-HT, receptor mRNA levels were measured using a ribonuclease protection assay, and the data are expressed as means ± standard errors of three determinations. Similar results have been observed in two additional experiments.

**TABLE I**

| Treatment                          | 5-HT$_{2A}$ receptor mRNA
|------------------------------------|-----------------------------|
| Vehicle                           | 100.0 ± 4.5                |
| 5-HT                              | 174.0 ± 4.3$^*$             |
| Actinomycin D                     | 31.8 ± 2.3$^*$              |
| Actinomycin D + 5-HT              | 32.0 ± 2.0$^*$              |

$^* p < 0.001$, versus vehicle.

Effects of acute exposure to 5-HT on 5-HT$_{2A}$ receptor mRNA levels (Fig. 3), the effects on receptor density of brief receptor stimulation were studied. Receptor down-regulation activated by a 15-min exposure to 5-HT (stippled bars) continued after the addition of the receptor antagonist ketanserin. The delayed receptor down-regulation proceeded to an extent similar to that seen in cells continually exposed to 5-HT. Cells treated with ketanserin had the same density of receptors as control cells confirming that the down-regulation observed in cells treated with both 5-HT and ketanserin was not due to the presence of residual ketanserin in the binding assay.

**DISCUSSION**

A major finding in the current study is that agonists elicit a biphasic change in levels of mRNA encoding 5-HT$_{2A}$ receptors. Acute exposure to 5-HT leads to an increase in receptor mRNA levels whereas longer exposure causes receptor mRNA levels to return to control values. Levels of cyclophilin mRNA were not altered indicating that the effects of 5-HT were not the result of a generalized change in the transcriptional activity of P11 cells. A transient increase in $\alpha$_-adrenergic receptor mRNA levels has also been observed after exposure to agonists. However, in contrast to the current results in which a decrease in levels of receptor mRNA to below control levels was not observed, prolonged exposure to agonists has been reported to reduce $\beta$-adrenergic receptor density on cells treated with 10 $\mu$M 5-HT decreased as a function of time of exposure to drug (Fig. 10, solid bars). Using a paradigm similar to that used to study greater than in cells treated with 5-HT alone (Fig. 9); the half-lives ($t_{1/2}$) of receptors were 136 and 230 min, respectively (Fig. 9, inset). In three experiments ($n = 9$), actinomycin D accelerated 5-HT-induced down-regulation by 26–41% (34 ± 4%, $p < 0.01$).

Effects of Acute Exposure to 5-HT on the Density of 5-HT$_{2A}$ Receptors—Receptor density on cells treated with 10 $\mu$M 5-HT decreased as a function of time of exposure to drug (Fig. 10, solid bars). Using a paradigm similar to that used to study...
Regulation of 5-HT2A Receptor mRNA

FIG. 10. Effects of acute exposure to 5-HT on the density of 5-HT2A receptors. P11 cells were incubated with 10 μM 5-HT (solid bars), 1 μM ketanserin (dotted line), or ketanserin (open bars) for the times indicated. Total incubation times for cells treated with 5-HT or 5-HT and then ketanserin were identical. Cells were harvested as indicated, membranes were prepared, and binding assays carried out using 1 nM [3H]-LSD. Results were calculated as a percentage of radioligand specifically bound to membranes of vehicle-treated cells, each assayed simultaneously and in triplicate. Data are combined from two experiments and are shown as means ± standard errors (n = 8). Similar results have been obtained in a third experiment (n = 5). *p < 0.001, versus untreated control (dotted bar).

adrenergic receptor mRNA levels to 50% of control values (17).

Surprisingly, exposure to 5-HT for periods of time as short as 1 min was sufficient to cause a delayed increase in 5-HT2A receptor mRNA levels. The finding that prolonged exposure to 5-HT is not required to alter receptor mRNA levels demonstrates that increases in levels of receptor mRNA do not require prolonged stimulation of the receptors and indicates that exposure to 5-HT for short periods of time is sufficient to trigger increases in receptor mRNA. A process that requires only brief receptor stimulation might be expected to regulate receptor mRNA in vivo, where the duration of action of endogenous 5-HT is transient, as is its resynthesis and metabolism. In addition, effects on receptor mRNA, stimulation of receptors for brief periods of time also has marked effects on receptor density (see below).

A recently identified AP-1-binding site in the promoter region of the 5-HT2A receptor gene (30) raised the possibility that immediate early genes such as c-fos participated in triggering the increase in levels of receptor mRNA. However, instead of an increase in the rate of transcription of the 5-HT2A receptor gene a transient increase in the stability of receptor mRNA was observed. Induction of receptor mRNA via a post-transcriptional mechanism which increases receptor mRNA stability is unique among non-peptide G protein-coupled receptors. Thus, for β2 (17) and α1-adrenergic (13) receptors, as well as for 5-HT2A receptors in uterine smooth muscle cells (24), enhanced rates of receptor gene transcription appear to account for agonist-induced increases in receptor mRNA.

Similar to the results of studies of β2-adrenergic receptor mRNA (17), 5-HT2A receptor mRNA levels were elevated for only a short period of time after exposure to agonists, a finding that appears to be a consequence of a return of the half-life of receptor mRNA to control values during prolonged exposure to 5-HT. The decline in mRNA levels was not secondary to oxidation or metabolism of 5-HT because 5-HT, which has a half-life of approximately 13 h in medium, was added every 12 h. Decreases in the stability of receptor mRNA transcripts after prolonged exposure to agonists have been documented in other receptor systems (19, 31), suggesting that changes in receptor mRNA stability are a common way to decrease levels of mRNA encoding neurotransmitter receptors.

A 32-kDa mRNA-binding protein, which has a binding specificity for repeats of the AUUUA nucleotide pentamer, a consensus sequence that has been implicated in regulation of mRNA stability, has been described for β2- and β3-adrenergic receptor mRNA (32). The expression of this protein varies inversely with β2-adrenergic receptor mRNA levels suggesting that it binds to, and destabilizes, β2-adrenergic receptor mRNA. Whether or not related stabilizing or destabilizing proteins that affect the stability of 5-HT2A receptor mRNA exist, and whether or not exposure to 5-HT would alter their activity, is not known at the present time.

Results from the current study contrast with results of two recent studies of the regulation of 5-HT2A receptor mRNA. In rat cerebellar granule cells (23) and in rat uterine smooth muscle cells (24), exposure to 5-HT causes a 2–4-fold elevation of 5-HT2A receptor mRNA levels that is sustained for 12 h or more, whereas in P11 cells receptor mRNA was elevated for only a brief period of time. The disparate results might be a consequence of distinct regulatory mechanisms. Inhibition of de novo protein synthesis with cycloheximide prevents agonist-induced increases in levels of 5-HT2A receptor mRNA in uterine smooth muscle cells (24) and in cerebellar granule cells (23), but did not prevent the increase in P11 cells. Furthermore, the increase in 5-HT2A receptor mRNA in uterine smooth muscle cells appears to be the result of an increase in the rate of transcription of the receptor gene (24), rather than a change in the stability of receptor mRNA as was observed in P11 cells. Together these findings indicate that multiple mechanisms can participate in regulating levels of 5-HT2A receptor mRNA and that these regulatory mechanisms may be cell type-specific.

Hydrolysis of phosphoinositides appears to be a critical step in a pathway which regulates expression of 5-HT2A receptor mRNA in P11 cells. Partial agonists at stimulating PI hydrolysis were also partial agonists at increasing 5-HT2A receptor mRNA. In addition, stimulation of PI turnover through activation of α1-adrenergic receptors on P11 cells was found to increase levels of 5-HT2A receptor mRNA. Moreover, the effects of 5-HT on levels of receptor mRNA were dose dependent and comparable EC50 values for PI hydrolysis and mRNA increase (approximately 400 nm) were observed.2

The finding that stimulation of α1-adrenergic receptors modulated 5-HT2A receptor mRNA expression indicates that cross-talk from α1-adrenergic to 5-HT2A receptors occurs in P11 cells. Coupling to the PI hydrolysis cascade, a signaling pathway that is common to a variety of receptors including 5-HT2A and α1-adrenergic receptors, could explain the shared ability of these heterologous receptor systems to alter 5-HT2A receptor mRNA levels. Regulation of 5-HT2A receptor mRNA resulting from stimulation of α1-adrenergic receptors demonstrates that non-serotonergic systems coupled to PI turnover can alter expression of 5-HT2A receptor mRNA and raises the interesting possibility that levels of 5-HT2A receptor mRNA can be regulated through activation of heterologous receptor systems coupled to stimulation of phospholipase C. Potential effects of 5-HT2A receptor activation on regulating the expression of α1-adrenergic receptor mRNA were not investigated. Because homologous regulation of mRNA encoding 5-HT2A receptors in P11 cells involves activation of protein kinase C (see below), 5-HT2A receptor-mediated regulation of α1-adrenergic receptor mRNA is likely to occur since protein kinase C-mediated increases in α1-adrenergic receptor mRNA levels have been reported elsewhere (13).

Protein kinase C is directly (via diacylglycerol) and indirectly (via inositol trisphosphate-induced calcium release) activated

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2 R. C. Ferry, unpublished observations.
by metabolites of PI turnover. Stimulation of protein kinase C, which is required for agonist-promoted changes in levels of mRNA encoding thyrotropin-releasing hormone and α₁β-adrenergic receptors, resulted in a large increase in levels of 5-HT₂A receptor mRNA, whereas inhibition of protein kinase C with a selective inhibitor blocked PMA- and 5-HT-induced increases in receptor mRNA levels. These findings indicate that the effects of 5-HT on receptor mRNA required activation of protein kinase C. Because activation of protein kinase C also leads to desensitization of 5-HT₂A receptors (33), it appears that protein kinase C is involved in control of 5-HT₂A receptor-effector coupling, as well as expression of 5-HT₂A receptor mRNA. Previous investigations (9, 11) have shown that exposure of P11 cells to 5-HT leads to down-regulation of 5-HT₂A receptors, a finding that contrasts with the 5-HT-induced increase in levels of receptor mRNA required for agonist-promoted changes in levels of 5-HT₂A receptors. However, inhibition of 5-HT-promoted receptor down-regulation resulted in a large increase in levels of 5-HT₂A receptor mRNA. Because exposure to 5-HT appears to trigger events which lead to desensitization of 5-HT₂A receptors (33), it might be expected to have effects on receptor density in vivo where the duration of receptor occupancy is limited by the short half-life of the endogenous neurotransmitter.

In summary, the present results demonstrate that heterologous receptor systems coupled to stimulation of PI hydrolysis alter expression of 5-HT₂A receptor mRNA through a post-transcriptional mechanism requiring activation of protein kinase C, that brief receptor engagement has significant effects on receptor mRNA levels and receptor density, and that increases in levels of receptor mRNA have a modulatory effect on receptor expression. These findings suggest a novel possibility for regulation of 5-HT₂A receptors by protein kinase C.

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