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Regulation of the 5-Hydroxytryptamine$_{1B}$ Receptor in Opossum Kidney Cells after Exposure to Agonists

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SUMMARY

The density of 5-hydroxytryptamine (5-HT)$_{1B}$ receptors and their coupling to the inhibition of cAMP accumulation were investigated in opossum kidney cells maintained in culture. The density and properties of the receptor were determined using [^{125}I] iodocyanopindolol as the radioligand. The pharmacological specificity of the binding site was consistent with that expected for a 5-HT$_{1B}$ receptor. Serotonin inhibited forskolin-stimulated cAMP accumulation with an EC_{50} of 4–8 nM. Compounds known to show selectivity at the 5-HT$_{1B}$ receptor, such as trifluoromethylphenylpiperazine and CGS-12066B, also inhibited forskolin-stimulated cAMP accumulation, acting as full agonists with efficacies comparable to that of serotonin. Other β-adrenergic receptor antagonists, including (−)-pindolol and (−)-alprenolol, bound to the receptor with high affinity and acted as partial agonists capable of inhibiting forskolin-stimulated cAMP accumulation. Exposure of cells to 5-HT resulted in a time- and dose-dependent decrease in the density of 5-HT$_{1B}$ receptors that was not accompanied by a change in the K_d of the binding site for [^{125}I] iodocyanopindolol. A maximum decrease of 60% in the number of 5-HT$_{1B}$ receptors was evident after a 16-hr treatment with 1 μM 5-HT. Concomitant with the observed decrease in the density of receptors was a marked increase in the EC_{50} for 5-HT-mediated inhibition of forskolin-stimulated cAMP accumulation. The EC_{50} was increased 4–5-fold after a 16-hr exposure to 1 μM 5-HT, and the maximal level of inhibition was markedly decreased. Whereas pretreatment with moderate concentrations of 5-HT (100–300 nM) for 16 hr produced significant decreases in the density of 5-HT$_{1B}$ receptors and increases in the EC_{50} for inhibition of forskolin-stimulated cAMP formation, there was little change in the maximal level of inhibition that could be attained. Such a combination of changes could be explained by the presence of "spare" 5-HT$_{1B}$ receptors on these cells.

Serotonin (5-HT) mediates a wide range of physiological responses, acting through multiple subtypes of receptor. Abnormalities in this neurotransmitter system have been implicated in a variety of psychiatric disorders, including depression, schizophrenia, anxiety, anorexia nervosa, and obsessive-compulsive disorder. It has recently become apparent that the effects of many drugs with neuropsychopharmacological activity may be attributed, at least in part, to their ability to modulate the level of serotonergic neurotransmission in the central nervous system.

Several pharmacologically distinct classes of receptors for 5-HT have been defined. These subtypes were initially characterized on the basis of their differential affinity for [^{3}H]5-HT. The 5-HT$_1$ subclass included those receptors that demonstrated nanomolar affinity for [^{3}H]5-HT, whereas the 5-HT$_2$ subclass had a lower affinity for [^{3}H]5-HT. The existence of multiple subtypes of 5-HT$_1$ receptors was first proposed on the basis of the biphasic inhibition of the binding of [^{3}H]5-HT by spiperone (1). At present there are at least six subtypes of 5-HT receptor, five of which couple to intracellular second messenger systems through G proteins; the other, the 5-HT$_3$ receptor, is a ligand-gated ion channel (2). G protein-coupled receptors include the 5-HT$_{1A}$, 5-HT$_{1B}$, and 5-HT$_{1D}$ receptor subtypes, which couple to inhibition or, in some cases, to stimulation of cAMP accumulation (3–7), and the 5-HT$_{1C}$ and 5-HT$_2$ receptors, which couple to stimulation of phosphatidylinositol hydrolysis (8–13). Although the 5-HT$_{1C}$ receptor was initially assigned to the 5-HT$_1$ subclass because of its affinity for 5-HT, it is now apparent that this receptor is more closely related to the 5-HT$_2$ receptor, based on the predicted amino acid sequences of the two receptor proteins, their similar pharmacological profiles, and the fact that they both couple to the same second messenger (11–13). The 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors exhibit a similar pharmacological profile and until recently were thought to be species variants of the same receptor, with the 5-HT$_{1B}$ receptor being found in rats, mice, and opossum and the 5-HT$_{1D}$ receptor in cat, guinea pig, bovine, pig, and human brain. Recent molecular cloning studies not only have identified the mRNA encoding

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)-tetralin; OK, opossum kidney; [^{125}I]ICYP, [^{125}I]iodocyanopindolol; MEM, minimal essential medium; TFMPP, trifluoromethylphenylpiperazine; CGS-12066B, 7-trifluoromethyl-4(4-methyl-1-piperazinyl)pyrrolo[1,2-*a*]quinoxaline, 1:2 maleate; PBS, phosphate-buffered saline; G protein, guanine nucleotide-binding protein.

the human 5-HT$_{1D}$ and 5-HT$_{1B}$ receptors but also have indicated that mRNA for both subtypes of receptor is present in the rat (14–18).

Pharmacological differentiation of the 5-HT receptor subtypes is based on their relative affinities for a variety of agents. The 5-HT$_{1A}$ receptor can be differentiated from the 5-HT$_{1B}$ and 5-HT$_{1D}$ subtypes on the basis of its relative affinity for the agonists 8-OH-DPAT and BMY 7378 and for the antagonist spiperone. The closely related 5-HT$_{1B}$ and 5-HT$_{1D}$ subtypes can be distinguished by the higher relative affinity of the 5-HT$_{1B}$ receptor for RU24969 and (−)-pindolol. Few compounds possess true selectivity for specific 5-HT receptor subtypes, but subtypes can be identified by their relative pharmacological specificity. Several of the compounds that have been used to distinguish different subtypes of 5-HT receptor also bind to receptors for other biogenic amines. For example, spiperone is an antagonist at the dopamine D2 receptor and (−)-pindolol at the β-adrenergic receptor. This cross-reactivity probably reflects the close similarity between 5-HT receptors and other receptors that are members of the G protein-linked family of receptors. This similarity has, in fact, been used in several cases to clone the genes that encode these receptor proteins. For example, the 5-HT$_{1A}$ receptor was cloned by virtue of its sequence homology with β-adrenergic receptors. The human 5-HT$_{1D}$ receptor has also been cloned, and its sequence has been utilized to identify 5-HT$_{1B}$- and 5-HT$_{1D}$-like receptors from rat tissues (14–18).

The regulation of 5-HT$_{1B}$ receptors has been studied, and in several cases anomalous or ambiguous results have been obtained. Lesions of serotonergic pathways after administration of 5,7-dihydroxytryptamine do not cause detectable changes in 5-HT$_{1B}$ receptors in rat forebrain and have been reported to up-regulate, down-regulate, or not affect the density of 5-HT$_{1B}$ receptors in rat substantia nigra (19, 20). Interpretation of results from studies of the regulation of 5-HT$_{1B}$ receptors after chemical destruction of serotonergic neurons is further complicated by the fact that receptors may be located on both pre- and postsynaptic elements. Autoreceptors on serotonergic terminals in rat brain include 5-HT$_{1B}$ receptors (21), but 5-HT$_{1B}$ receptors are also found at postsynaptic sites (19, 20). Loss of presynaptic terminals would be expected to decrease the density of receptors localized to those terminals, but an increase in postsynaptic receptors could occur in response to decreased serotonergic activation.

Cell lines maintained in culture represent an alternative to *in vivo* model systems for the study of receptor regulation. Such cell lines have been utilized to study the regulation of 5-HT$_2$ and 5-HT$_{1C}$ receptors (22–26). The 5-HT$_{1B}$ receptor has been detected on Chinese hamster lung fibroblasts (CCL 39 cells) and on an epithelial cell line from oppossum kidney (OK cells). Coupling of the 5-HT$_{1B}$ receptor to inhibition of cAMP accumulation was also demonstrated in OK cells (7). OK cells have also been used to study the regulation of α_2-adrenergic (27) and dopamine D1 receptors (28).

Experimental Procedures

Materials. MEM with Earle's balanced salts and L-glutamine was obtained from JRH Biosciences (Lenexa, KS). Penicillin (10,000 units/ml), streptomycin (10,000 μg/ml), and trypsin-EDTA were obtained from GIBCO Laboratories (Grand Island, NY). Cyanopindolol was a gift from Günter Engel (Sandoz, Basel, Switzerland); it was iodinated by using chloramine T and purified by paper chromatography. Sodium [^{125}I]iodide and [^{3}H]adenine were obtained from New England Nuclear (Boston, MA). TFMPP, CGS-12066B, ketanserin, and 8-OH-DPAT were obtained from Research Biochemicals Inc. (Natick, MA). Serotonin (creatinine sulfate complex), GTP, and (−)-alprenolol were supplied by Sigma Chemical Co. (St. Louis, MO). The water-soluble forskolin derivative 7-deacetyl-6-(*N*-acetylglycyl)forskolin was obtained from Calbiochem (San Diego, CA), and 3-isobutyl-1-methylxanthine was obtained from Aldrich Chemical Co. (Milwaukee, WI).

Cell culture. OK cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in monolayer culture in medium composed of MEM with Earle's balanced salts and L-glutamine, 10% (v/v) serum (Hyclone, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin. The serum was pretreated with 5% (w/v) activated charcoal, which removed >99% of the endogenous serotonin, resulting in a maximal final concentration of serotonin in freshly prepared medium of approximately 3 nM. Cells were maintained at 37° in a humidified atmosphere containing 10% CO_2.

For studies of receptors, cells were plated at a density of 10^6 cells/15-cm culture plate, in 25 ml of medium. For measurement of cAMP accumulation, cells were plated at a density of 10^4 cells/well in 24-well culture plates containing 0.5 ml of medium. Medium was replaced every 3–4 days, and experiments were carried out after 7–14 days in culture.

Membrane preparation. Culture plates were placed on ice and the medium was removed. Cells were then washed with 10 ml of ice-cold PBS (composed of 138 mM NaCl, 4 mM KCl, 5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 11 mM glucose) before being scraped into 10 ml of PBS and transferred to a 40-ml centrifuge tube. Residual cells remaining attached to plates were scraped into an additional 5 ml of PBS and pooled with the original material. Cells from two 15-cm plates were pooled and centrifuged at 20,000 × *g* for 15 min at 5°. The pellet was resuspended by homogenization (Brinkmann Polytron, setting 6 for 10 sec) in 30 ml of 5 mM Tris, 5 mM EDTA, pH 7.4, and was centrifuged at 20,000 × *g* for 20 min at 5°. The resulting pellet was resuspended by homogenization in 2 ml of 50 mM Tris, pH 7.4, transferred to glass vials, frozen rapidly in a mixture of dry ice and ethanol, and stored at −80°.

Assays with [^{125}I]ICYP. Aliquots of crude membranes, prepared as described above, were thawed, homogenized in 30 ml of 50 mM Tris, pH 7.4, and centrifuged at 20,000 × *g* for 20 min at 5° before being resuspended in 50 mM Tris, pH 7.4, containing 1 mM $MgCl_2$, with or without 0.3 mM GTP, for use in Scatchard analyses or competition studies.

In experiments in which cells had been treated with 5-HT$_{1B}$ agonists, an additional washing step was incorporated. Thawed membranes were resuspended by homogenization in 30 ml of 50 mM Tris, pH 7.4, containing 1 mM $MgCl_2$ and 0.3 mM GTP, and were incubated at 37° for 45 min before being centrifuged at 20,000 × *g* for 20 min at 5°. The pellet was homogenized in an additional 30 ml of ice-cold 50 mM Tris, pH 7.4, centrifuged as described above, and resuspended in 50 mM Tris, pH 7.4, containing 1 mM $MgCl_2$ and 0.3 mM GTP. The K_d for [^{125}I]ICYP was the same when determined with membranes from untreated cells and with membranes from cells that had been pretreated with 5-HT or other agonists.

For Scatchard analysis, [^{125}I]ICYP was included at final ligand concentrations of 5–200 pM. Assays were initiated by addition of a suspension of OK cell membranes (15–40 μg of protein), followed by incubation at room temperature for 120 min. Assays were terminated by the addition of 8 ml of ice-cold wash buffer (10 mM Tris, 100 mM NaCl, pH 7.4), and samples were rapidly filtered. Filters were washed with an additional 16 ml of wash buffer. [^{125}I]ICYP retained on filters was measured by using a gamma counter. Total assay volume was 200 μl/tube, and nonspecific binding was determined in parallel incubations containing 10 μM 5-HT. Data were transformed using Scatchard analysis to calculate K_d and B_{max} values.

For studies of the inhibition of binding of [^{125}I]ICYP by competing

drugs, OK cell membranes (15–40 μg of protein) and radioligand (20 pM) were incubated in the presence of increasing concentrations of a competing ligand. Assays were performed in 50 mM Tris, pH 7.4, containing 1 mM $MgCl_2$. Samples were incubated, filtered, and washed as described above.

In some experiments, binding was carried out at two concentrations of radioligand. OK cell membranes (15–40 μg of protein) from individual samples were incubated with 5 pM and 25 pM [^{125}I]ICYP for 120 min at room temperature, as described above. Specific binding was determined for each concentration of radioligand. Results were calculated as a percentage of the specific binding detected in membranes from control cells incubated with the same concentrations of radioligand and assayed simultaneously. A mean of these percentage of control values for the 5 pM and 25 pM concentrations of [^{125}I]ICYP was then calculated for each sample. For individual samples, there was good agreement between the two percentage of control values obtained. This served as an internal control in each experiment, confirming that the washing procedure had effectively removed the exogenous 5-HT from each sample.

Measurement of cAMP accumulation. OK cells growing as monolayers on 24-well culture plates were incubated with 1 μCi of [^{3}H] adenine for 2 hr, to radiolabel intracellular stores of ATP (7, 29). Cells were washed with 0.5 ml of MEM to remove extracellular radioactivity and were incubated for 2 min at 37° in 0.5 ml of MEM containing 1 mM 3-isobutyl-1-methylxanthine, to inhibit phosphodiesterase activity. cAMP accumulation was then stimulated by addition of 10 μM forskolin, either alone or premixed with varying concentrations of 5-HT or other compounds active at the 5-HT_{1B} receptor. After 5 min the plate was transferred onto ice, medium was removed by aspiration, and 1 ml of ice-cold 5% (w/v) trichloroacetic acid was added. The trichloroacetic acid extracts were then centrifuged at 10,000 × *g* for 5 min at 5°, to remove cell debris, before the samples were chromatographed using a combination of Dowex 50W-X4 cation exchange resin and neutral alumina AG-7, to isolate [^{3}H]cAMP and [^{3}H]ATP. Radioactivity in these two fractions was determined by liquid scintillation counting, and the data were transformed to express cAMP formation as a percentage of the [^{3}H]ATP in each sample. EC_{50} values were calculated using a log-logit transformation of the inhibition of cAMP accumulation, followed by linear regression analysis. Maximal inhibition of cAMP accumulation was observed in the presence of 1 μM 5-HT.

Protein content. Protein content was assayed by the method of Lowry *et al.* (30), using bovine serum albumin as the standard.

Results

Characterization of [^{125}I]ICYP binding. Scatchard analysis of the binding of [^{125}I]ICYP to OK cell membranes revealed a single class of sites that could be converted from high to low affinity by the inclusion of 0.3 mM GTP. The density of 5-HT_{1B} receptors in OK cell membranes, measured either in the presence or in the absence of GTP, was routinely 30–50 fmol/mg of protein, and K_d values were 6–10 pM and 20–30 pM for the high and low affinity sites, respectively (Fig. 1).

Competition curves for inhibition of the binding of [^{125}I]ICYP demonstrated that the receptor had nanomolar affinity for 5-HT (Fig. 2). Two other compounds that are relatively selective for the 5-HT_{1B} receptor subtype, TFMPP and CGS-12066B, had affinities for this receptor in the submicromolar range, with TFMPP being more potent than CGS-12066B. The β-adrenergic receptor antagonists (−)-alprenolol and (−)-pindolol (Fig. 2) also demonstrated a high affinity for this receptor, comparable to that seen with 5-HT, whereas isoproterenol showed a low affinity, inhibiting about 50% of specific binding of [^{125}I]ICYP at a concentration of 100 μM (data not shown). 8-OH-DPAT and ketanserin, which are selective for the 5-HT_{1A} and 5-HT_2 receptors, respectively, showed low affinity for the binding site for [^{125}I]ICYP on OK cells (Fig. 2).

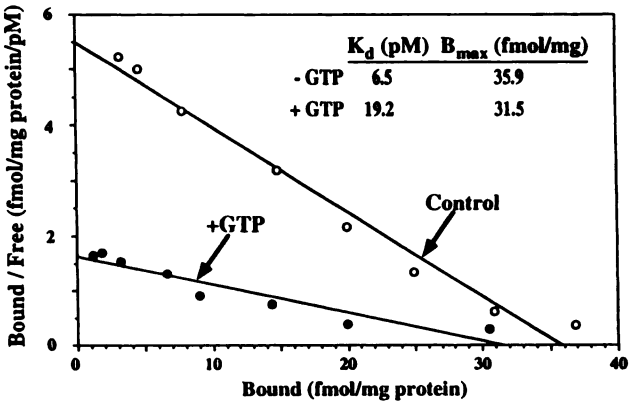

Fig. 1. Binding of [^{125}I]ICYP to membranes from OK cells in the presence or absence of GTP. Radioligand binding assays were performed as described in Experimental Procedures, in the presence (●) or absence (○) of 0.3 mM GTP.

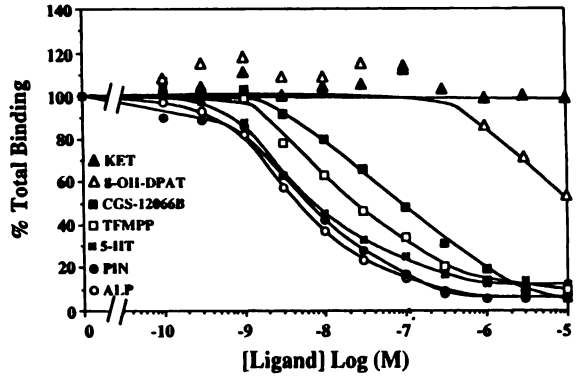

Fig. 2. Inhibition of binding of [^{125}I]ICYP by agonists and antagonists. Radioligand binding assays were conducted as described in Experimental Procedures. Each *point* is the mean of triplicate determinations. *KET*, ketanserin; *PIN*, (−)-pindolol; *ALP*, (−)-alprenolol.

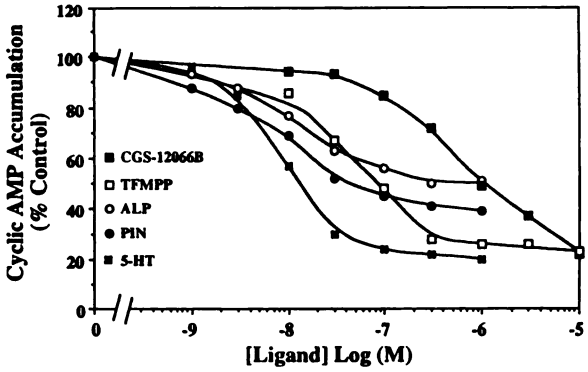

Fig. 3. Inhibition of forskolin-stimulated cAMP accumulation in OK cells. cAMP accumulation assays were performed as described in Experimental Procedures. Each *point* is the mean of triplicate determinations. *PIN*, (−)-pindolol; *ALP*, (−)-alprenolol.

Inhibition of forskolin-stimulated cAMP accumulation in OK cells. 5-HT was a potent inhibitor of forskolin-stimulated cAMP accumulation in OK cells, with an EC_{50} of 4–8 nM (Fig. 3). Maximal inhibition of 70–80% was achieved with concentrations of 5-HT of 100 nM or greater. Two other compounds, TFMPP and CGS-12066B, which had been previously identified as being relatively selective 5-HT_{1B} receptor

agonists, were also full agonists at this receptor, with EC_{50} values of 40 nM and 500 nM, respectively. The β-adrenergic receptor antagonists (−)-pindolol and (−)-alprenolol were partial agonists in this system, causing maximal inhibition of forskolin-stimulated cAMP accumulation of 50–60%.

Down-regulation of 5-HT$_{1B}$ receptors after exposure to 5-HT. Exposure of OK cells to a high concentration of 5-HT (10 μM) for 16 hr resulted in a 60% decrease in the density of 5-HT$_{1B}$ receptors, as measured by the binding of [^{125}I]ICYP. Scatchard analysis (Fig. 4) showed that this decrease in receptor density was not accompanied by a change in K_d for the radioligand, confirming that the washing protocol efficiently removed the 5-HT. A time course study of exposure to 10 μM 5-HT showed a 40% reduction in the number of 5-HT$_{1B}$ receptors after 4 hr. A maximal decrease in the number of 5-HT$_{1B}$ receptors occurred within 8 hr, and the decrease was maintained for at least an additional 10 hr (Fig. 5). Concomitant with the decrease in the number of 5-HT$_{1B}$ receptors was a time-dependent increase in the EC_{50} for 5-HT-mediated inhibition of forskolin-stimulated cAMP accumulation (Fig. 5, *inset*). The decrease in receptors after exposure to 5-HT was concentration dependent, with a maximal depletion of 60% being induced by 1 μM 5-HT (Fig. 6). A concentration-dependent increase in the

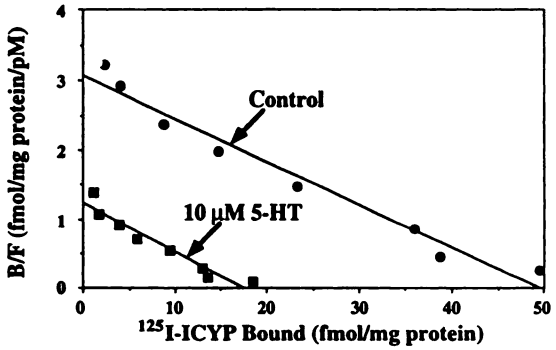

Fig. 4. Effect of exposure to 5-HT on 5-HT$_{1B}$ receptors. Cells were treated with 10 μM 5-HT for 16 hr. Radioligand binding assays were performed as described in Experimental Procedures. These Scatchard plots are representative of four such determinations.

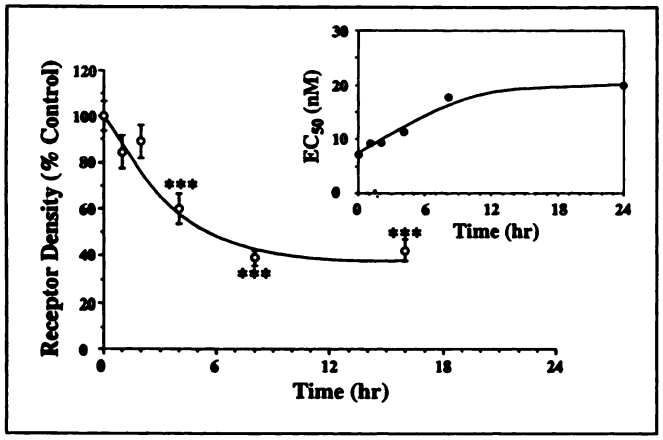

Fig. 5. Time-dependent decrease in density of 5-HT$_{1B}$ receptors and increase in EC_{50} (*inset*) for 5-HT-mediated inhibition of forskolin-stimulated cAMP accumulation. OK cells were exposed to 10 μM 5-HT for various times, after which samples were processed for radioligand binding and cAMP accumulation assays, as described in Experimental Procedures. Data presented are means ± standard errors (four to eight determinations). Statistical comparisons were made with control cells using analysis of variance. ***, $p < 0.005$.

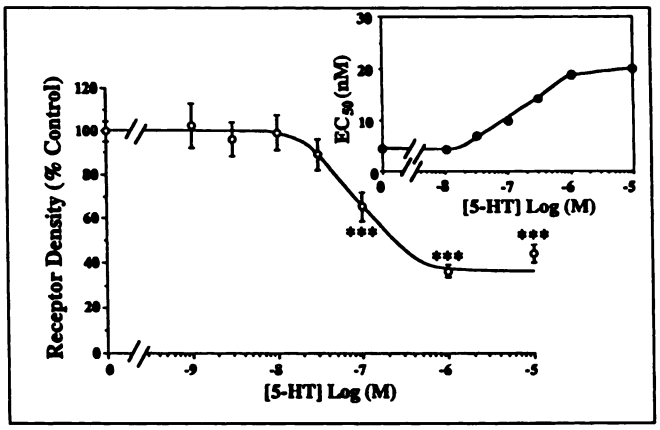

Fig. 6. Concentration-dependent decrease in the density of 5-HT$_{1B}$ receptors and increase in EC_{50} (*inset*) for 5-HT-mediated inhibition of forskolin-stimulated cAMP accumulation. OK cells were pretreated with various concentrations of 5-HT for 16 hr. The density of 5-HT receptors was calculated from [^{125}I]ICYP binding data and the EC_{50} was determined from cAMP accumulation assays, as described in Experimental Procedures. The data are pooled from two experiments, each performed in quadruplicate. Statistical comparisons were made with control cells by using analysis of variance. ***, $p < 0.005$.

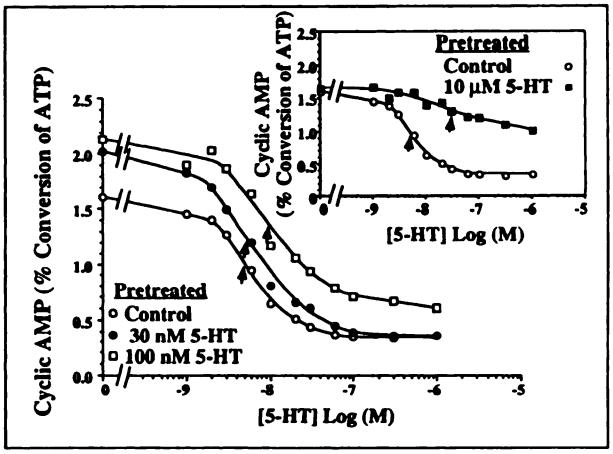

Fig. 7. Effect of pretreatment with 5-HT on 5-HT-mediated inhibition of forskolin-stimulated cAMP accumulation. OK cells were pretreated with 30 nM, 100 nM, or 10 μM (*inset*) of 5-HT for 16 hr. The inhibition of forskolin-stimulated cAMP accumulation was assayed as described in Experimental Procedures. Data represent the means of quadruplicate determinations and are representative of two such experiments. *Arrows*, calculated EC_{50} values.

EC_{50} for 5-HT-mediated inhibition of forskolin-stimulated cAMP accumulation was also observed (Fig. 6, *inset*).

Exposure of OK cells to 5-HT, at concentrations of 30–300 nM, for 16 hr consistently resulted in a marked increase in the amount of cAMP generated during a subsequent 5-min stimulation with 10 μM forskolin (Fig. 7). This sensitization did not appear to affect the calculated EC_{50} values, because there was no difference in the EC_{50} for 5-HT-mediated inhibition of cAMP accumulation between control cells and cells treated with 30 nM 5-HT, despite the sensitization to forskolin (Fig. 7). Exposure to 30 nM or 100 nM 5-HT induced similar degrees of sensitization of forskolin-stimulated cAMP accumulation. There was a significant increase in EC_{50} in cells treated with 100 nM 5-HT (Fig. 7). Pretreatment with higher concentrations of 5-HT (1–10 μM) also resulted in a sensitization to forskolin, a response that showed a marked time dependence. Sensitiza-

tion was maximal after 8 hr and had returned close to control levels after 16–24 hr (data not shown). Although exposure of OK cells to high concentrations of 5-HT (1–10 μM) for 16 hr did not cause sensitization of forskolin-stimulated cAMP formation, it did result in a significant increase in the EC_{50} for 5-HT-mediated inhibition of this response (Figs. 6 and 7, *insets*). Maximal inhibition by 5-HT was not reduced after incubation of cells with 30 nM 5-HT, despite an increase in the sensitivity to forskolin (Fig. 7). Higher concentrations of 5-HT resulted in a progressive decrease in the ability of 5-HT to inhibit cAMP accumulation (Fig. 7 and *inset*).

Sensitization of forskolin-stimulated cAMP accumulation complicates calculation of the maximal inhibition that can be achieved. The absolute amount of cAMP formation inhibited was increased after pretreatment with low concentrations of 5-HT but was decreased after pretreatment with 1–10 μM 5-HT (Table 1). If the data are presented as the percentage of inhibition of the forskolin stimulation, then the maximal inhibition was decreased after exposure to 5-HT at concentrations of 100 nM and above (Table 1).

Down-regulation of 5-HT_{1B} receptors by other agonists. The relatively selective 5-HT_{1B} receptor agonist TFMPP was also capable of inducing a decrease in the number of 5-HT_{1B} receptors on OK cells after a 16-hr exposure (Fig. 8). This decrease in receptor density also occurred without any change in the affinity of the receptor for [^{125}I]ICYP (data not shown).

Discussion

OK cells represent a useful model system with which to study the regulation of 5-HT_{1B} receptors after exposure to agonists. The present results confirm previous findings that this cell line expresses measurable levels of 5-HT_{1B} receptors (B_{max} = 30–50 fmol/mg of protein), as quantitated by [^{125}I]ICYP binding (7). This receptor has a high affinity (6–10 pM) for [^{125}I]ICYP in the absence of GTP. Addition of GTP resulted in a decrease in the affinity of the receptor for [^{125}I]ICYP (K_d = 20–30 pM), with no change in the number of binding sites. Characterization of 5-HT_{1B} receptors in an earlier passage of OK cells revealed a density of 88 fmol/mg of protein, with a K_d of 47 pM for [^{125}I]ICYP, measured in the presence of GTP. In the absence of GTP, the binding of [^{125}I]ICYP was better fit by a two-site model, with affinities of 10 pM and 345 pM (7). The discrepancy between our results and those of Murphy and Bylund (7) may reflect the use of a passage of OK cells different from that used in the present study. Several lines of OK cells exist that differ with respect to the types of receptors they express; the line used in this study does not express α_2-adrenergic, dopamine D1, or parathyroid hormone receptors (7, 27, 28). Alternatively, the differences may be attributable to differences in culture conditions. The fetal bovine serum used in the present study was routinely treated with activated charcoal to remove endogenous 5-HT; this treatment may also remove other low molecular weight substances normally present in complete serum. The content of serum in the culture medium (10%) was higher than that in previous studies, and serum was present throughout the entire course of the experiment. The line of OK cells used in the present study demonstrated consistent levels of 5-HT_{1B} receptor expression through a large number of passages.

TABLE 1

Effect of 5-HT pretreatment on the maximal inhibition of forskolin-stimulated cAMP accumulation

OK cells were treated with varying concentrations of 5-HT for 16 hr, after which cAMP accumulation assays were carried out as described in Experimental Procedures. The data from two experiments were pooled, and statistical comparisons were made with control cells by using analysis of variance. *n*, number of determinations.

5-HT pretreatment	Maximal inhibition of cAMP accumulation		*n*
	Net inhibition of cAMP formation	Inhibition	
	% conversion of ATP	%	
0	1.24 ± 0.03	77.63 ± 0.59	11
30 nM	1.56 ± 0.07[a]	79.81 ± 1.31	7
100 nM	1.42 ± 0.08[b]	72.70 ± 0.59[a]	7
300 nM	1.34 ± 0.15	67.39 ± 1.17[a]	3
1 μM	0.98 ± 0.09[c]	60.66 ± 1.97[a]	3
10 μM	0.70 ± 0.06[a]	44.20 ± 3.86[a]	7

[a] $p < 0.005$.
[b] $p < 0.05$.
[c] $p < 0.01$.

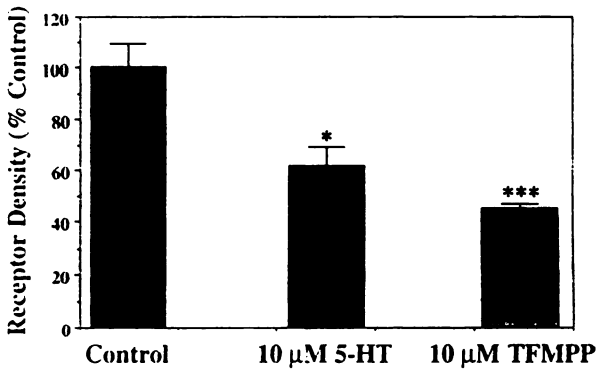

Fig. 8. Decrease in the density of 5-HT_{1B} receptors after exposure to 5-HT or TFMPP. OK cells were treated with 10 μM 5-HT or 10 μM TFMPP for 16 hr, after which membranes were prepared and receptor number was quantitated from [^{125}I]ICYP binding data, as described in Experimental Procedures. Neither treatment produced any change in K_d for this radioligand. Data are means ± standard errors (four experiments). Statistical comparisons with control cells were made by using analysis of variance. *, $p < 0.05$; ***, $p < 0.005$.

The competition curves for [^{125}I]ICYP showed that the 5-HT_{1B} agonists 5-HT, TFMPP, and CGS-12066B exhibited the expected order of potency. In contrast, 5-HT_{1A}- and 5-HT_2-selective compounds had a low affinity for this binding site, whereas the two β-adrenergic receptor antagonists (−)-pindolol and (−)-alprenolol had a high affinity for the receptor. These findings are consistent with the supposition that the binding site is a 5-HT_{1B} receptor. Functional studies of the coupling of this receptor to inhibition of cAMP accumulation were also consistent with the presence of a 5-HT_{1B} receptor on OK cells. The 5-HT_{1B} agonists 5-HT, TFMPP, and CGS-12066B were full agonists in this system and showed the expected order of potency, similar to that observed in competition studies for binding of ^{125}I-ICYP. Two β-adrenergic receptor antagonists, (−)-pindolol and (−)-alprenolol, were partial agonists. The activity of these classical β-adrenergic receptor antagonists was consistent with results of binding data with [^{125}I]ICYP, in which a decrease in affinity in the presence of GTP was observed, suggesting that [^{125}I]CYP is also an agonist at this receptor.

The response exhibited by OK cells after exposure to 5-HT includes at least four components, (i) a decrease in receptor density, (ii) an increase in the EC_{50} for 5-HT-mediated inhibition of forskolin-stimulated cAMP accumulation, (iii) a de-

crease in the maximal inhibition of cAMP formation that can be elicited by 5-HT, and (iv) sensitization of the adenylyl cyclase system to a subsequent stimulation by forskolin. Exposure of OK cells to low concentrations of 5-HT (~30 nM) for 16 hr had no significant effect on the density of 5-HT$_{1B}$ receptors and did not result in any change in the EC$_{50}$. The maximum level of 5-HT-mediated inhibition of forskolin-stimulated cAMP accumulation was not decreased, but a marked sensitization of the adenylyl cyclase system to forskolin was seen. Treatment with intermediate concentrations of 5-HT (100–300 nM) for 16 hr resulted in a significant decrease in the number of 5-HT$_{1B}$ receptors, a marked increase in the EC$_{50}$ for 5-HT-mediated inhibition of cAMP accumulation, either a slight decrease or no change in the maximal levels of inhibition, and a sensitization to forskolin similar in magnitude to that seen in cells pretreated with 30 nM 5-HT. The combination of a decrease in the density of receptors, an increase in EC$_{50}$, and no change in maximal response may be explained by the presence of "spare" 5-HT$_{1B}$ receptors. A 16-hr exposure to high concentrations of 5-HT (1–10 μM) led to an additional small reduction in the number of 5-HT$_{1B}$ receptors and an increase in the EC$_{50}$ for 5-HT-mediated inhibition of forskolin-stimulated cAMP accumulation. A marked decrease in the maximal level of inhibition of forskolin-stimulated cAMP accumulation was also observed. This decrease was apparent when the results were expressed either as the absolute amount of inhibition or as the percentage of inhibition. These changes can be explained by a combination of down-regulation and desensitization of the 5-HT$_{1B}$ receptor upon exposure to high concentrations of agonist. The additional marked decrease in the maximal inhibition of forskolin-stimulated cAMP accumulation after treatment with high concentrations of 5-HT may be explained by a decrease in the density of receptors sufficient to deplete the receptor reserve present in naive cells.

The EC$_{50}$ for down-regulation, approximately 100 nM 5-HT, was 1 order of magnitude higher than the EC$_{50}$ for inhibition of forskolin-stimulated cAMP accumulation. Although this may reflect a distinction between these two responses in the degree of receptor activation required to achieve these changes, instability of 5-HT in culture medium may also contribute to the observed difference. Exposing OK cells to the 5-HT$_{1B}$ receptor agonist TFMPP also resulted in a significant decrease in the density of 5-HT$_{1B}$ receptors. The effects of TFMPP on the other responses seen after treatment with 5-HT have not been investigated.

The results obtained in the present study suggest that the 5-HT$_{1B}$ receptor in OK cells down-regulates and desensitizes in response to agonist only after prolonged exposure to relatively high concentrations of 5-HT, concentrations that are sufficient to maximally activate the receptor, as determined by inhibition of forskolin-stimulated cAMP accumulation. In this respect, the 5-HT$_{1B}$ receptor differs from other biogenic amine receptors that also couple to inhibition of adenylyl cyclase but demonstrate more rapid desensitization after exposure to agonist. For example, desensitization of the α_2-adrenergic receptor in OK cells was half-maximal 1 min after exposure to 10 μM epinephrine (31).

Consistent with the present findings, agonists at several other receptors that couple to inhibition of cAMP accumulation have also been reported to mediate sensitization to forskolin (32–38). The mechanism involved in this sensitization has not been elucidated, but it appears to represent a compensatory process exhibited by cells in response to exposure to agonists coupled to inhibition of cAMP formation.

Data from another study of the regulation of 5-HT$_{1B}$ receptor levels and receptor coupling to inhibition of adenylyl cyclase in OK cells have recently been reported (39). Compared with the data presented here, those investigators reported a slightly higher 5-HT$_{1B}$ receptor density (64 fmol/mg of protein) and a markedly lower affinity for [^{125}I]ICYP binding to the 5-HT$_{1B}$ receptor (216 pM). Although 5-HT-mediated down-regulation of the 5-HT$_{1B}$ receptor was observed in that study, it occurred to a lesser degree (maximal decrease of 40%) and more slowly (10–20% decrease after 6 hr), compared with our findings. Another noticeable difference between these two studies is the reduced potency of 5-HT for inhibition of forskolin-stimulated cAMP accumulation (EC$_{50}$ = 166 nM, compared with 4–8 nM in the present study). Despite these quantitative differences, the regulatory responses of the 5-HT$_{1B}$ receptor after exposure to 5-HT are qualitatively similar in both studies.

In vivo investigations of the regulatory mechanisms associated with activation of 5-HT$_{1B}$ receptors have generated contradictory results (19, 20). The present findings clearly demonstrate that OK cells respond to 5-HT$_{1B}$ receptor agonists with a decrease in receptor density and an increase in the concentration of 5-HT required to inhibit cAMP formation. These observations indicate that the OK cell represents an excellent model system for further investigation of the mechanisms involved in the regulation of these receptors.

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References

1. Pedigo, N. W., H. I. Yamamura, and D. L. Nelson. Discrimination of multiple [^{3}H]5-hydroxytryptamine binding sites by the neuroleptic spiperone in rat brain. *J. Neurochem.* **36**:220–226 (1981).
2. Frazer, A., S. Maayani, and B. B. Wolfe. Subtypes of receptors for serotonin. *Annu. Rev. Pharmacol. Toxicol.* **30**:307–348 (1990).
3. De Vivo, M., and S. Maayani. Characterization of the 5-hydroxytryptamine$_{1A}$ receptor-mediated inhibition of forskolin-stimulated adenylate cyclase activity in guinea pig and rat hippocampal membranes. *J. Pharmacol. Exp. Ther.* **238**:248–253 (1986).
4. Barbaccia, M. L., N. Brunello, D.-M. Chuang, and E. Costa. Serotonin-elicited amplification of adenylate cyclase activity in hippocampal membranes from adult rat. *J. Neurochem.* **40**:1671–1679 (1983).
5. Shenker, A., S. Maayani, H. Weinstein, and J. P. Green. Two 5-HT receptors linked to adenylate cyclase in guinea pig hippocampus are discriminated by 5-carboxamidotryptamine and spiperone. *Eur. J. Pharmacol.* **109**:427–429 (1985).
6. Fargin, A., J. R. Raymond, J. W. Regan, S. Cotecchia, R. J. Lefkowitz, and M. G. Caron. Effector coupling mechanisms of the cloned 5-HT1A receptor. *J. Biol. Chem.* **264**:14848–14852 (1989).
7. Murphy, T. J., and D. B. Bylund. Characterization of serotonin-1B receptors negatively coupled to adenylate cyclase in OK cells, a renal epithelial cell line from the opossum. *J. Pharmacol. Exp. Ther.* **249**:535–543 (1989).
8. Conn, P. J., and E. Sanders-Bush. Agonist-induced phosphoinositide hydrolysis in choroid plexus. *J. Neurochem.* **47**:1754–1760 (1986).
9. Conn, P. J., and E. Sanders-Bush. Regulation of serotonin-stimulated phosphoinositide hydrolysis: relation to the serotonin 5-HT-2 binding site. *J. Neurosci.* **6**:3669–3675 (1986).
10. de Chaffoy de Courcelles, D., J. E. Leysen, F. De Clerck, H. Van Belle, and P. A. J. Janssen. Evidence that phospholipid turnover is the signal transducing system coupled to serotonin-S$_2$ receptor sites. *J. Biol. Chem.* **260**:7603–7608 (1985).
11. Pritchett, D. B., A. W. J. Bach, M. Wozny, O. Taleb, R. Dal Toso, J. C. Shih, and P. H. Seeburg. Structure and functional expression of cloned rat serotonin 5HT-2 receptor. *EMBO J.* **7**:4135–4140 (1988).
12. Julius, D., A. B. MacDermott, R. Axel, and T. M. Jessell. Molecular characterization of a functional cDNA encoding the serotonin 1c receptor. *Science* (Washington D. C.) **241**:558–564 (1988).
13. Julius, D., K. N. Huang, T. J. Livelli, R. Axel, and T. M. Jessell. The 5HT2 receptor defines a family of structurally distinct but functionally conserved serotonin receptors. *Proc. Natl. Acad. Sci. USA* **87**:928–932 (1990).

14. Hamblin, M. W., and M. A. Metcalf. Primary structure and functional characterization of a human 5-HT_{1D}-type serotonin receptor. *Mol. Pharmacol.* **40**:143–148 (1991).
15. Hamblin, M. W., and M. A. Metcalf. Cloning of the human 5-HT_{1D} serotonin receptor gene and its rat homolog. *Soc. Neurosci. Abstr.* **17**:719 (1991).
16. Branchek, T., N. Adham, M. Macchi, P. Hartig, and R. Weinshank. Expression and characterization of a cloned rat 5-HT_{1B} receptor: comparison with a cloned human 5-HT_{1D} receptor. *Soc. Neurosci. Abstr.* **17**:719 (1991).
17. Adham, N., P. Romanienko, P. Hartig, R. L. Weinshank, and T. Branchek. The rat 5-hydroxytryptamine$_{1B}$ receptor is the species homologue of the human 5-hydroxytryptamine$_{1D\beta}$ receptor. *Mol. Pharmacol.* **41**:1–7 (1992).
18. Voigt, M. M., D. J. Laurie, P. H. Seeberg, and A. Bach. Molecular cloning and characterization of a rat brain cDNA encoding a 5-hydroxytryptamine$_{1B}$ receptor. *EMBO J.* **10**:4017–4023 (1991).
19. Vergé, D., G. Daval, M. Marcinkiewicz, A. Patey, S. El Mestikawy, H. Gozlan, and M. Hamon. Quantitative autoradiography of multiple 5-HT_1 receptor subtypes in the brain of control or 5,7-dihydroxytryptamine-treated rats. *J. Neurosci.* **6**:3474–3482 (1986).
20. Offord, S. J., G. A. Ordway, and A. Frazer. Application of [^{125}I]iodocyanopindolol to measure 5-hydroxytryptamine$_{1B}$ receptors in the brain of the rat. *J. Pharmacol. Exp. Ther.* **244**:144–153 (1988).
21. Engel, G., M. Göthert, D. Hoyer, E. Schlicker, and K. Hillenbrand. Identity of inhibitory presynaptic 5-hydroxytryptamine (5-HT) autoreceptors in the rat brain cortex with 5-HT_{1B} binding sites. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **332**:1–7 (1986).
22. Ivins, K. J., and P. B. Molinoff. Receptors for monoamines on cultured cells. *Am. J. Hypertens.* **3**:43S–46S (1990).
23. Ivins, K. J., and P. B. Molinoff. Serotonin-2 receptors coupled to phosphoinositide hydrolysis in a clonal cell line. *Mol. Pharmacol.* **37**:622–630 (1990).
24. Pauwels, P. J., P. Van Gompel, and J. E. Leysen. Rapid desensitization and resensitization of 5-HT_2 receptor mediated phosphatidylinositol hydrolysis by serotonin agonists in quiescent calf aortic smooth muscle cells. *Life Sci.* **47**:2009–2019 (1990).
25. Tsutsumi, M., M. K. Skinner, and E. Sanders-Bush. Transferrin gene expression and synthesis by cultured choroid plexus epithelial cells: regulation by serotonin and cyclic adenosine 3′,5′-monophosphate. *J. Biol. Chem.* **264**:9626–9631 (1989).
26. Burris, K. D., M. Breeding, and E. Sanders-Bush. (+)Lysergic acid diethylamide, but not its nonhallucinogenic congeners, is a potent serotonin 5HT_{1C} receptor agonist. *J. Pharmacol. Exp. Ther.* **258**:891–896 (1991).
27. Murphy, T. J., and D. B. Bylund. Characterization of *alpha*-2 adrenergic receptors in the OK cell, an opossum kidney cell line. *J. Pharmacol. Exp. Ther.* **244**:571–578 (1988).
28. Bates, M. D., M. G. Caron, and J. R. Raymond. Desensitization of DA_1 dopamine receptors coupled to adenylyl cyclase in opossum kidney cells. *Am. J. Physiol.* **260**:F937–F945 (1991).
29. Shimizu, H., J. W. Daly, and C. R. Creveling. A radioisotopic method for measuring the formation of adenosine 3′,5′-cyclic monophosphate in incubated slices of brain. *J. Neurochem.* **16**:1609–1619 (1969).
30. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
31. Jones, S. B., S. L. Leone, and D. B. Bylund. Desensitization of the *alpha*-2 adrenergic receptor in HT29 and opossum kidney cell lines. *J. Pharmacol. Exp. Ther.* **254**:294–300 (1990).
32. Nathanson, N. M., W. L. Klein, and M. Nirenberg. Regulation of adenylate cyclase activity mediated by muscarinic acetylcholine receptors. *Proc. Natl. Acad. Sci. USA* **75**:1788–1791 (1978).
33. Heisler, S., and C. B. Srikant. Somatostatin-14 and somatostatin-28 pretreatment down-regulate somatostatin-14 receptors and have biphasic effects on forskolin-stimulated cyclic adenosine 3′,5′-monophosphate synthesis and adrenocorticotropin secretion in mouse anterior pituitary tumor cells. *Endocrinology* **117**:217–225 (1985).
34. Sabol, S. L., and M. Nirenberg. Regulation of adenylate cyclase of neuroblastoma × glioma hybrid cells by α-adrenergic receptors. II. Long-lived increase of adenylate cyclase activity mediated by α receptors. *J. Biol. Chem.* **254**:1921–1926 (1979).
35. Hoffman, B. B., H. Chang, E. Dall'Aglio, and G. M. Reaven. Desensitization of adenosine receptor-mediated inhibition of lipolysis: the mechanism involves the development of enhanced cyclic adenosine monophosphate accumulation in tolerant adipocytes. *J. Clin. Invest.* **78**:185–190 (1986).
36. Bates, M. D., S. E. Senogles, J. R. Bunzow, S. B. Liggett, O. Civelli, and M. G. Caron. Regulation of responsiveness at D_2 dopamine receptors by receptor desensitization and adenylyl cyclase sensitization. *Mol. Pharmacol.* **39**:55–63 (1991).
37. Jones, S. B., M. L. Toews, J. T. Turner, and D. B. Bylund. α_2-Adrenergic receptor-mediated sensitization of forskolin-stimulated cyclic AMP production. *Proc. Natl. Acad. Sci. USA* **84**:1294–1298 (1987).
38. Jones, S. B., and D. B. Bylund. Characterization and possible mechanisms of α_2-adrenergic receptor-mediated sensitization of forskolin-stimulated cyclic AMP production in HT29 cells. *J. Biol. Chem.* **263**:14236–14244 (1988).
39. Pleus, R. C., and D. B. Bylund. Desensitization and down-regulation of the 5-hydroxytryptamine$_{1B}$ receptor in the opossum kidney cell line. *J. Pharmacol. Exp. Ther.* **261**:271–277 (1992).

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