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8-Hydroxy-2-(di-*n*-propylamino)tetralin-Responsive 5-Hydroxytryptamine$_4$-like Receptor Expressed in Bovine Pulmonary Artery Smooth Muscle Cells

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SUMMARY

Bovine pulmonary artery smooth muscle (SM) cells express a novel 5-hydroxytryptamine (5-HT) (5-HT$_4$-like) receptor coupled to cAMP accumulation. cAMP radioimmunoassay established the agonist and antagonist profiles of this receptor. 5-HT (EC_{50} = 91 ± 33 nM) and 5-methoxytryptamine were equipotent at the SM cell 5-HT receptor and both were more potent than 5-carboxamidotryptamine. Other tryptamine derivatives were less potent but remained full agonists. These findings are consistent with previous reports regarding 5-HT$_4$ and 5-HT$_4$-like receptors in the central nervous system. The most potent antagonists were the antidepressant compounds nortriptyline (IC_{50} = 177 ± 153 nM) and zimelidine (IC_{50} = 202 ± 101 nM). The 5-HT$_3$ and 5-HT$_4$ antagonist 3-tropanyl-indole-3-carboxylate (ICS 205–930) was also a competitive antagonist at this 5-HT$_4$-like receptor (pA_2 = 6.3). Antagonist affinities differed slightly at the SM cell receptor, compared with other 5-HT$_4$ and 5-HT$_4$-like receptors in the central nervous system. Nonetheless, the SM cell 5-HT$_4$-like receptor displayed the same differential antagonist potencies as reported for these other receptors (ICS 205–930 > MDL 72222 and mianserin > ketanserin). 8-Hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) was the most potent agonist for this 5-HT$_4$-like receptor (EC_{50} = 6.4 ± 3.4 nM). 8-OH-DPAT-induced cAMP accumulation could be blocked by ICS 205–930 but not by the 5-HT$_{1A}$ antagonist 1-(2-methoxyphenyl)-4-[4-(2-pthalimido)butyl]piperazine hydrobromide, distinguishing the SM cell 5-HT receptor from 5-HT$_{1A}$ receptors. The mechanism of 5-HT-stimulated cAMP production was also investigated. First, GTP augmented basal and 5-HT-stimulated cAMP accumulation. Second, antisera to the carboxyl terminus of the α subunit of G_s, attenuated 5-HT-mediated adenylate cyclase activation. This established that 5-HT-stimulated cAMP accumulation in SM cells required G_s. These findings suggest that SM cells express a novel 5-HT$_4$-like receptor positively coupled to adenylate cyclase. An unexpected finding was that 8-OH-DPAT is a potent partial agonist. These studies suggest that there may be heterogeneity among 5-HT$_4$-like receptors.

Numerous receptor binding sites exist for serotonin (5-HT) in the central nervous system and in the periphery (1). Pharmacologic analysis and radioligand binding studies have provided evidence for distinct receptor subtypes. To date, these include at least the 5-HT$_{1A}$ (2, 3), 5-HT$_{1B}$ (4), 5-HT$_{1C}$ (5), 5-HT$_{1D}$ (6), 5HT$_2$ (7), and 5-HT$_3$ (8, 9) receptors, in addition to the recently described 5-HT$_4$ receptors (10).[1]

The primary signal transduction pathways initiated by these receptor subtypes also serve to categorize them further. Both 5-HT$_{1C}$ and 5-HT$_2$ receptors stimulate phospholipase C and phosphoinositide hydrolysis (11, 12). 5-HT$_3$ receptors, unique from all other 5-HT receptors because of their lack of G protein coupling, serve as gates for ion channels (13). 5-HT$_{1A}$, 5-HT$_{1B}$, and 5-HT$_{1D}$ receptors all inhibit adenylate cyclase (14–16). Finally, 5-HT$_4$ receptors stimulate adenylate cyclase (10).

Serotonin has been implicated in a number of vasoactive phenomena including mitogenesis and the maintenance of vascular SM tone (17, 18). These processes have not been wholly attributed to any of the classical 5-HT receptors except for the involvement of 5-HT$_2$ receptors modulating contraction/relaxation responses. Evidence indicates that serotonin may regulate some of these vasoactive responses via a 5-HT$_4$ receptor in bovine pulmonary artery SM cells (19). Specifically, 5-HT

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[1] The term "5-HT$_4$ receptor" remains under debate. The appelation has been tentatively accepted by the Serotonin Club Receptor Nomenclature Committee as of October 1991, as appeared in Ref. 47.

ABBREVIATIONS: 5-HT, serotonin; SM, smooth muscle; G protein, guanine nucleotide-binding protein; G_s, stimulatory guanine nucleotide-binding protein; G_o, predominant pertussis toxin-sensitive guanine nucleotide-binding protein expressed in brain; 8-OH-DPAT, (±)-8-hydroxy-2-(di*n*-propylamino)tetralin; ICS 205–930, 3-tropanyl-indole-3-carboxylate; 5-MT, 5-methoxytryptamine; 5-CT, 5-carboxamidotryptamine; 5-BT, 5-benzyloxytryptamine; 5-HIAA, 5-hydroxyindole-3-acetic acid; DOI, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl; TFMPP, *m*-trifluoromethylphenylpiperazine; MDL 72222, 3-tropanyl-3,5-dichlorobenzoate; NAN-190, 1-(2-methoxyphenyl)-4-[4-(2-pthalimido)butyl]piperazine hydrobromide; PBS, phosphate-buffered saline; NCB.20 cell, human neuroblastoma cell; LSD, lysergic acid diethylamide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; RIA, radioimmunoassay.

directly effects configurational changes in SM cells through a receptor associated with increased cAMP production (20). We, therefore, examined primary cultures of these cells for the presence of a 5-HT_4 or 5-HT_4-like receptor and investigated the mechanism by which it may stimulate cAMP accumulation in these cells.

Materials and Methods

Reagents. RPMI 1640 medium, fungizone (amphotericin B), Dulbecco's PBS, penicillin G potassium, and streptomycin were purchased from GIBCO Laboratories (Grand Island, NY). 5-HT creatine sulfate, 5-CT maleate, (±)-DOI, 8-OH-DPAT hydrobromide, TFMPP HCl, ketanserin tartrate, MDL72222, metoclopramide, mianserin HCl, forskolin, haloperidol, pindolol, ICS 205–930, zimelidine HCl, cyproheptadine HCl, diltiazem HCl, prochlorperazine dimaleate, 1-(*m*-chlorophenyl)-biguanide HCl, and NAN-190 were all obtained from Research Biochemicals Incorporated (Natick, MA). Tryptamine, *N*-ω-methyltryptamine, 5-BT HCl, 5-HIAA, *N,N*-dimethyl-5-methoxytryptamine, 5-hydroxy-*N*-ω-methyltryptamine oxalate, and DL-5-hydroxytryptophan were obtained from Aldrich Chemical Company (Milwaukee, WI). 5-MT and isobutylmethylxanthine were purchased from Sigma Chemical Co. (St. Louis, MO). Methysergide maleate was obtained from Sandoz (Basel, Switzerland) and methiothepine was from BIOMOL Research Laboratories (Plymouth Meeting, PA). Nortriptyline HCl was obtained from Eli Lilly and Company (Indianapolis, IN).

[α-^{32}P]ATP was purchased from New England Nuclear Research Products (Boston, MA). ^{125}I-cAMP was synthesized by Dr. Thomas Gettys (Durham, NC) (21). RM-1 antibody (anti-$G_{s\alpha}$) and GO-1 antibody (anti-$G_{o\alpha}$) were also obtained from New England Nuclear Research Products (Boston, MA). Anti-succinyl-cAMP antibody (rabbit antiserum) was purchased from Dr. Kevin Martin (St. Louis University, St. Louis, MO).

Cell culture. Primary cultures of bovine pulmonary artery SM cells were performed as previously described (19). Cells were grown in 75-cm^2 flasks in RPMI 1640 medium containing 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1.25 μg/ml amphotericin B at 37° in 5% CO_2/95% O_2. Media were changed every 2–3 days until cells reached confluence. For cAMP RIA determination cells were split into 12-well culture plates, incubated under the same conditions, and fed with the same frequency.

cAMP RIA. Confluent SM cells in 12-well culture plates were washed with 1 ml of prewarmed PBS and isobutylmethylxanthine (500 μM) three times and preincubated in the same solution for 30 min at 37°. Tryptamine, *N*-ω-methyltryptamine, 5-HIAA, forskolin, haloperidol, and ICS 205–930 were dissolved in ethanol. 5-BT was dissolved in methanol, and DL-5-hydroxytryptophan was dissolved in 0.5 N HCl. All of the remaining compounds were dissolved in water. Concentrations of ethanol, methanol, and 0.5 N HCl never exceeded 1% and these solvents did not alter cAMP levels. 5-HT or other agents were added to the cells for a 10-min incubation at 37°. The solution was aspirated and the reaction was terminated with the addition of 250 μl of 0.6 M perchloric acid to each well. Cells were then incubated at 4° for 50 min. Two hundred microliters of this preparation were neutralized with 50 μl of 3 M $KHCO_3$. Samples were then frozen at −20° or immediately suspended in 20 mM MES buffer, pH 6.2. cAMP was then assayed without acetylation using ^{125}I-cAMP and anti-succinyl-cAMP antibody.

Adenylate cyclase assay. Confluent cells in 75-cm^2 flasks were incubated in serum-free medium at 37° for 1 hr. They were then washed twice with PBS and scraped with a rubber policeman after suspension in a solution containing 5 mM Tris and 2 mM EDTA, pH 7.4. This preparation was homogenized with five strokes of a Dounce homogenizer and then underwent centrifugation at 33,000 × *g* for 20 min. The pellet was resuspended in buffer containing 7.5 mM Tris, 1.25 mM $MgCl_2$, and 2 mM EDTA and was either placed on ice or incubated for 30 min at 37° in 1/1 dilution with buffer containing 2.7 mM phosphoenolpyruvate, 0.05 mM GTP, 0.1 mM cAMP, 0.12 mM ATP, 0.05 IU/ml myokinase, and 0.01 IU/ml pyruvate kinase. The final assay (50 μl) thus consisted of 3 mM Tris, 410 mM $MgCl_2$, 1.2 mM EDTA, 2.7 mM phosphoenolpyruvate, 0.05 mM GTP, 0.1 mM cAMP, 0.12 mM ATP, 0.05 IU/ml myokinase, 0.01 IU/ml pyruvate kinase, and 5 μCi/ml [α-^{32}P]ATP. The reaction was initiated by addition of the membrane preparation and was incubated for 30 min at 30°. When antagonist compounds were utilized, the particle preparation was preincubated with the agent or buffer alone for 5 min on ice. When antisera were evaluated, either anti-$G_{s\alpha}$ or anti-$G_{o\alpha}$ was added to the membrane preparation, in a 1/100 dilution, and preincubated with the membrane preparation for 30 min before initiation of the assay. The reaction was halted by the addition of 1 ml of stop solution containing 0.4 mM ATP, 0.3 mM cAMP, and [^{3}H]cAMP (~25,000 cpm). Each condition was repeated in triplicate. cAMP formed during the reaction was extracted by the method of Salomon *et al.* (22) and calculated on a personal computer. Proteins were determined by the method of Bradford (23).

Equilibrium binding assays. Confluent cells were used to obtain membrane particle preparations by the methods described above. After centrifugation at 33,000 × *g* for 15 min, the membrane pellet was resuspended in buffer containing 100 mM NaCl and 50 mM Tris, pH 7.4, for the binding assay with [^{3}H]LSD, [^{3}H]8-OH-DPAT, and [^{3}H]5-HT, with or without 5-HT (10 μM). Particle preparations were incubated at 37° in a total volume of 1 ml and the assays were performed according to the protocol derived by Peroutka and Snyder (24).

Measurement of cytosolic calcium concentration. The cytosolic calcium concentration was determined in intact SM cells cultured on coverslips, using the intracellular fluorescent probe fura-2/acetoxymethylester (Calbiochem, La Jolla, CA). Cells were incubated at 37° with 250 μM Pluronic F-127 (BASF Corp., Wyandotte, MI) and 20 μM fura-2/acetoxymethyl ester in Hanks' balanced salt solution containing 0.01% bovine serum albumin, 1.3 mM calcium, and 10 mM HEPES, pH 7.4. Fluorescence measurements and calculations of cytosolic calcium were conducted with a fluorescence spectrometer (Perkin-Elmer, Norwalk, CT), by the method of Grynkiewicz *et al.* (25).

Measurement of phosphoinositide hydrolysis. SM cells grown in six-well plates were exposed to 5 μCi/ml *myo*-[^{3}H]inositol in their medium for 24 hr. Evaluation and measurement of inositol phosphate turnover were then undertaken according to the methods described by Martin (26).

Experimental design and data analysis. Agonist and antagonist dose-response curves were plotted as a function of the logarithm of the concentration of the drug. EC_{50} values report the agonist concentration necessary to stimulate 50% of the maximal activation, derived directly from each curve. IC_{50} values report the antagonist concentration necessary to decrease the response to 5 μM serotonin by 50% and were similarly calculated utilizing linear regression analysis (GraphPad, Inplot; GraphPAD Software, San Diego, CA). All experiments were conducted with at least five drug concentrations in duplicate or triplicate, unless otherwise noted. Data are presented as mean ± standard error of raw data or data normalized to the maximum 5-HT response, as described in the text or figure legends. The pA_2 value for ICS 205–930 was calculated by the method of Arunlakshana and Schild (27) from data presented in Fig. 3. Most statistics were performed using paired one- or two-tailed *t* test with Bonferroni correction. For Fig. 6, correlations were plotted with Statview (Abacus Concepts, Berkely, CA) using a linear regression model. Statistical significance was calculated from the *F* test of each of the regressions.

Results

Effect of serotonin and serotonin agonists on cAMP production in primary cultures of bovine pulmonary artery SM cells. A number of classic serotonergic agonists were tested by cAMP RIA for their ability to activate adenylate cyclase in primary cultures of bovine pulmonary artery SM cells. The pharmacologic characteristics of the 5-HT receptor

in SM cells appeared to be remarkably similar to those of the 5-HT_4 receptor in embryonic mouse colliculi neurons and the 5-HT_4-like receptor in NCB.20 cells (10, 28). Concentration-response curves for several of these agonists are shown in Fig. 1. The potencies and relative efficacies of the agents tested are shown in Table 1. Serotonin stimulated cAMP production 8.5-fold, with an EC_{50} of 91 ± 33 nM. The other tryptamine derivatives, including 5-MT, 5-CT, tryptamine itself, and 5-BT, were full or nearly complete agonists.

EC_{50} values for all of the tested tryptamine derivatives ranged from 90 nM to 44 μM. Notably, none of these compounds was significantly more potent than serotonin itself. As previously shown for a putative 5-HT_4-like receptor in NCB.20 cells, hydroxyl or methoxy substitutions at the 5-position of the indole ring in tryptamine conveyed greater potency to these compounds. More complex substitutions at the 5-position of the indole ring, however, reduced agonist affinity and *N*-methyl additions moderately decreased agonist efficacy. Additionally, neither 5-hydroxytryptophan nor 5-HIAA, the immediate precursor and product of serotonin metabolism, respectively, caused activation of adenylate cyclase.

Several agents outside of the tryptamine family of compounds were evaluated as well. Their actions at serotonin receptors have been documented previously. Two of the agents tested, including metoclopramide, one of the prokinetic substituted benzamide derivatives, acted as agonists in SM cells. As shown in Table 1, metoclopramide increased adenylate cyclase activity 2.5-fold, with an EC_{50} of 12.7 ± 3.7 μM. The low affinity of metoclopramide is completely consistent with its interaction at a 5-HT_4 receptor site (29). Interestingly, the tetralin derivative 8-OH-DPAT also stimulated the SM cell 5-HT receptor. This agent, classically associated with 5-HT_{1A} receptors, acted as a partial agonist and stimulated cAMP production 4.1-fold, with a mean EC_{50} of 6.4 ± 3.4 nM. This latter finding rendered 8-OH-DPAT the most potent agonist evaluated at the SM cell 5-HT receptor. Another 5-HT_1 receptor agonist, TFMPP, lacked any effect on cAMP accumulation in SM cells.

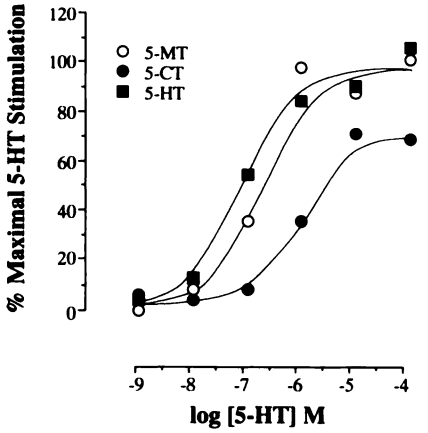

Fig. 1. Agonist concentration-response curves. cAMP RIAs were performed as described in Materials and Methods. Mean values from three or more experiments are shown. Data are displayed as percentage of maximal 5-HT stimulation. Each concentration was assayed at least in duplicate within each individual assay.

TABLE 1

Potencies and efficacies of serotonergic agonists

cAMP RIAs were performed as described in Materials and Methods. Five or more concentrations of each compound were tested. EC_{50} values were determined directly from semilogarithmic concentration-response curves. Values are the average ± standard error of raw data from duplicate, triplicate, or quadruplicate determinations.

Compound	EC_{50}	Fold stimulation
Tryptamines		
5-HT	91 ± 33 nM	8.3
5-MT	88 ± 40 nM	8.5
5-OH-*N*,ω-methyltryptamine	136 ± 66 nM	7.9
5-CT	2 ± 0.9 μM	5.6
N,*N*-Dimethyltryptamine	5 ± 1.6 μM	4.5
5-BT	10 ± 3.6 μM	10.2
Tryptamine	17 ± 6.4 μM	5.7
N,ω-Methyltryptamine	44 ± 28 μM	6.3
5-HIAA	No detectable stimulation	
5-Hydroxytryptophan	No detectable stimulation	
Other serotonergic agents		
8-OH-DPAT	6.4 ± 3.4 nM	4.1
DOI	No detectable stimulation	
TFMPP	No detectable stimulation	
Substituted benzamide derivatives		
Metoclopramide	13 ± 3.7 μM	2.4

Effects of serotonergic antagonists. A variety of agents were evaluated by cAMP RIA for their capacity to inhibit serotonin-stimulated cAMP accumulation in SM cells. The relative potencies of these compounds are listed in Table 2. The agents that displayed the greatest affinity for the SM cell receptor were nortriptyline, a tricyclic antidepressant, and zimelidine, a nontricyclic antidepressant. Both have demonstrated significant antagonist activity at serotonin transport sites (30). Imipramine, another tricyclic uptake inhibitor, was less potent than the aforementioned two compounds but did inhibit serotonin-stimulated cAMP production.

Mianserin and ketanserin, two agents that bind 5-HT_2 receptors preferentially, have previously been evaluated at the 5-HT_4-like receptor in NCB.20 cells (28). Both demonstrated lower affinities for the receptor in NCB.20 cells, compared with their affinities for classical 5-HT_2 receptors. Also, mianserin was a more potent antagonist at the receptor in NCB.20 cells

TABLE 2

Potencies of serotonergic antagonists

cAMP RIAs were performed as described in Materials and Methods. Five or more concentrations of each compound were tested in the presence of 5-HT (5 μM). IC_{50} values were determined directly from semilogarithmic concentration-response curves. Values are the average ± standard error of raw data from duplicate, triplicate, or quadruplicate determinations.

Compound	IC_{50}
Classical-serotonin antagonists	
ICS 205-930	250 ± 109 nM
Cyproheptadine	1.5 ± 0.4 μM
Mianserin	1.6 ± 0.6 μM
Methiothepine	3.9 ± 1.4 μM
MDL 72222	11.0 ± 4 μM
Methysergide	192 ± 51 μM
Ketanserin	200 ± 70 μM
NAN-190	No significant inhibition
Antidepressants	
Nortriptyline	177 ± 153 nM
Zimelidine	202 ± 101 nM
Imipramine	27 ± 15 μM
Antipsychotics	
Promethazine	1.7 ± 0.6 μM
Haloperidol	202 ± 101 μM

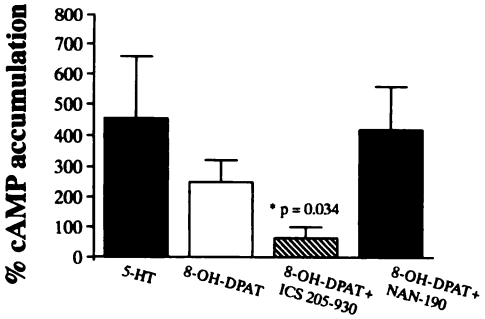

Fig. 2. Effects of 8-OH-DPAT, 5-HT, and antagonists on cAMP accumulation. Data were taken from cAMP RIAs performed as described in Materials and Methods. Values from three or more experiments (mean ± standard error) are shown for percentage of cAMP production for 5-HT (1 μM) and 8-OH-DPAT (1 nM). ICS 205–930 (1 μM) and NAN-190 (1 μM) alone did not affect basal levels of cAMP production (four experiments; data not shown). ICS 205–930 (1 μM) and NAN-190 (1 μM) were each tested in the presence of 8-OH-DPAT (1 nM). Each concentration was assayed at least in duplicate within each individual assay. *, paired one-tailed *t* test versus 8-OH-DPAT alone; Bonferroni correction used.

than was ketanserin. These compounds manifested similar pharmacologic characteristics at the 5-HT receptor in SM cells. Mianserin inhibited serotonin-mediated activation of adenylate cyclase 61 ± 6.3%, with an IC_{50} of 1.6 ± 0.6 μM. Ketanserin was markedly less potent, inhibiting cAMP accumulation 52 ± 17%, with an IC_{50} of 200 ± 70 μM. Thus, the relative affinities mianserin and ketanserin display for the 5-HT receptor in SM cells exclude interaction with a 5-HT_2 receptor and correlate with the rank order of potency these compounds manifest at the 5-HT receptor in NCB.20 cells.

Psychoactive compounds structurally similar to ketanserin have been shown to interact also at 5-HT_{1A} receptor sites. One such agent, haloperidol, was a poor antagonist at the 5-HT receptor in SM cells. It inhibited serotonin-stimulated cAMP production only 20.4 ± 3.9%, with an IC_{50} of 202 ± 101 μM. Interestingly, this is consistent with its rank order of activity at the 5-HT receptor in NCB.20 cells. Additionally, other psychoactive compounds such as promethazine demonstrated moderate antagonism at the 5-HT receptor in SM cell.

Two 5-HT_3 antagonists were examined because of their documented effects at 5-HT_4 receptors. ICS 205–930, with an IC_{50} of 250 ± 109 nM, was more potent than MDL72222, with an IC_{50} of 11 ± 4 μM, at the SM cell 5-HT receptor. Both compounds would be expected to be nearly equipotent at a 5-HT_3 receptor (31, 32). The rank order of potency of both of these agents at the SM cell 5-HT receptor is consistent with previous reports regarding these compounds at 5-HT_4 sites in the central nervous system (10, 33).

Importantly, ICS 205–930 also markedly antagonized the 8-OH-DPAT-mediated cAMP accumulation in SM cells, as shown in Fig. 2. This effect was specific, because ICS 205–930 did not block cAMP accumulation induced by adenosine, dopamine, or isoproterenol (three experiments; data not shown). Moreover, the 5-HT_{1A} antagonist NAN-190 demonstrated no inhibition of the 8-OH-DPAT response (four experiments, $p = 0.48$). In fact, NAN-190 appeared to augment measurable cAMP production in 8-OH-DPAT-stimulated SM cells. This was not, however, statistically significant.[2] These findings differentiate the SM cell 5-HT receptor from the 5-HT_{1A} receptor subtype, where NAN-190 would be expected to block the 8-OH-DPAT response. These data also further define the pharmacologic reactivity of the SM cell 5-HT receptor while accentuating the unique nature of this receptor, compared with classical 5-HT_{1A} receptors.

A summary evaluation of these studies clearly establishes the identity of the SM cell 5-HT receptor as most consistent with a 5-HT_4-like receptor. First, the tryptamine derivatives examined manifest affinities for the SM cell receptor that are strikingly similar to their documented affinities for other 5-HT_4 and 5-HT_4-like receptor sites (10, 28). Second, the rank orders of agonist and antagonist potency mirror previous findings for these agents at 5-HT_4 and 5-HT_4-like receptor sites. Subtle differences are apparent when absolute antagonist affinities for other congruent receptor sites and the SM cell receptor are compared. Nonetheless, a characteristic pharmacologic profile is present and it categorizes the SM cell receptor as a 5-HT_4 or 5-HT_4-like receptor.

Presence of serotonin-stimulated adenylate cyclase in bovine pulmonary artery SM cell membranes. Because we wanted to determine whether the 5-HT_4-like receptors in SM cells increased cAMP accumulation through a direct (membrane-delimited) or indirect (cytosolic) pathway, we examined the effects of 5-HT on washed membrane preparations derived from SM cells. This is shown in Fig. 3. The mean EC_{50} of 5-HT was 1.2 μM, whereas the maximum serotonin-stimulated response varied from 1- to 3.5-fold. In this assay 5-HT clearly stimulated adenylate cyclase activity, suggesting a direct (membrane-delimited) regulation of adenylate cyclase.

In order to further establish whether this effect was due to a 5-HT_4-like receptor, we performed the same assay in the presence of various concentrations of ICS 205–930. The serotonin

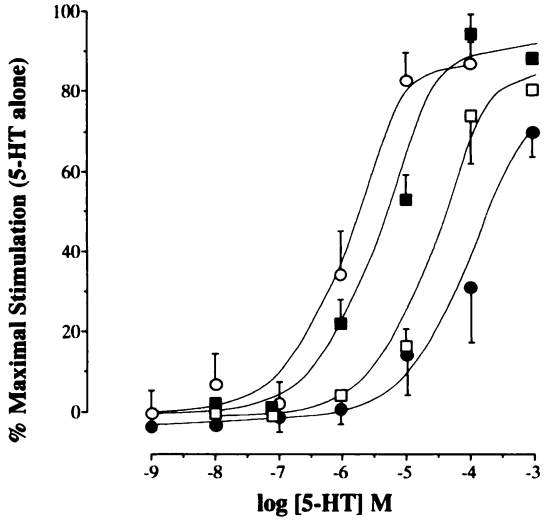

Fig. 3. Concentration-response curves for membrane adenylate cyclase assay. Assays were performed as described in Materials and Methods. Membrane particle preparations were preincubated with ICS 205–930 for 5 min and then exposed to 5-HT (■, 1 μM; □, 10 μM; ●, 100 μM). Data displayed are values from three to six experiments (mean ± standard error) for the calculated percentage of 5-HT stimulation. Each condition was assayed in duplicate or triplicate. The pA_2 value of 6.3 was obtained by the method of Arunlakshana and Schild (27) and correlated well with pK_B values calculated for each concentration of ICS 205–930 (6.22–6.31). The Schild plot was linear, suggesting that the experiments were performed at equilibrium. Individual assays averaged ≈13 pmol of cAMP/mg of protein.

[2] Although not statistically significant, the increase in cAMP accumulation induced by 8-OH-DPAT in the presence of NAN-190 suggests that a 5-HT_{1A} receptor that inhibits adenylate cyclase activity may be present in SM cells.

concentration-response curve displayed the expected parallel shift to the right in the presence of this antagonist. These results indicate that the demonstrated antagonism is competitive. Moreover, these results are entirely consistent with the previously documented effects of ICS 205–930 at the 5-HT_4-like receptor subtype (10, 33).

Effects of GTP on serotonin activation of adenylate cyclase. The aforementioned studies suggested the involvement of a G protein in the 5-HT_4-like receptor-mediated cAMP accumulation in SM cells. To test for the involvement of G proteins, we examined the effects of GTP on basal and 5-HT-stimulated adenylate cyclase activity. The serotonin response of washed membrane preparations displayed a clear dependence on the concentration of GTP in the assay mixture. The effect of increasing concentrations of GTP on basal adenylate cyclase activity and the activity observed in the presence of serotonin (10 μM) are displayed in Fig. 4. Increasing concentrations of GTP caused stimulation of basal adenylate cyclase activity and potentiated the response achieved by serotonin.

Inhibition of serotonin activation of adenylate cyclase by antibody to G_s. Data from the preceding studies strongly suggested that G_s played an integral intermediary role in serotonin-stimulated cAMP accumulation. To establish the role of the α subunit of G_s, we used antiserum to the carboxyl terminus of the α subunit of G_s (RM-1) to inhibit 5-HT receptor coupling to adenylyl cyclase (34). Preincubation with RM-1 markedly inhibited the serotonin-mediated activation of adenylate cyclase without affecting basal levels of cAMP. This is shown in Fig. 5. Antiserum to another G protein, G_o, and buffer alone did not inhibit the serotonin response or alter basal levels of cAMP.

Attempts to measure receptor binding using equilibrium binding assay. We were unable to quantitate specific binding data for [^{3}H]8-OH-DPAT, [^{3}H]LSD, or [^{3}H]5-HT, possibly due to a low density of receptors or the low affinity or low specific activity of available ligands (three experiments; data not shown).

Attempts to measure phosphoinositide hydrolysis. To ascertain whether the receptor in SM cells affected a second signal transduction pathway either as a primary event or as a result of activation of adenylate cyclase, we attempted to measured phosphoinositide hydrolysis in cells stimulated with serotonin, in concentrations ranging from 100 nM to 10 μM. No measurable inositol phosphate turnover was noted (two experiments; data not shown).

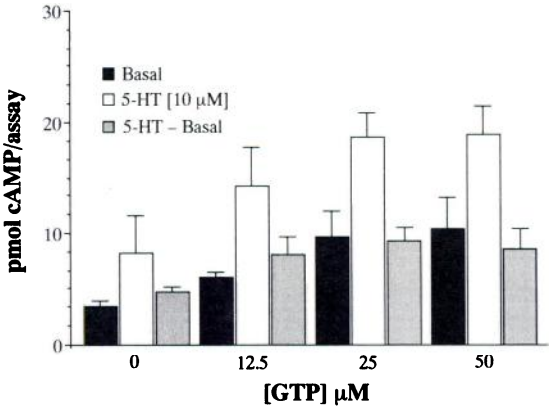

Fig. 4. Effect of increasing concentrations of GTP on cAMP accumulation. Membrane particle preparations were assayed in the presence of the noted concentration of GTP and in the presence (□) or absence (■) of 5-HT (10 μM). Results from three experiments (mean ± standard error) are shown for raw data. Differences between basal and 5-HT-stimulated conditions (□) were statistically significant ($p < 0.01$, paired one-tailed t test, Bonferroni correction).

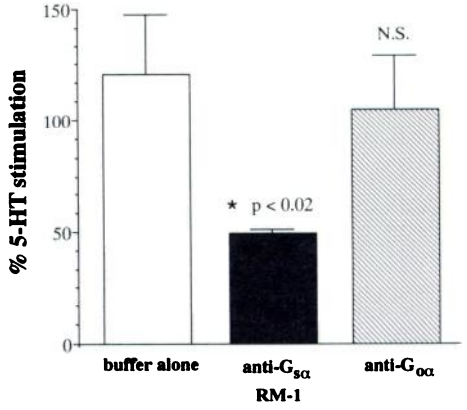

Fig. 5. Effect of $G_{s\alpha}$ antiserum on 5-HT-stimulated adenylate cyclase activity. Antisera to $G_{s\alpha}$ and $G_{o\alpha}$, at a 1/100 dilution, were preincubated with membrane particle preparations for 30 min before a membrane adenylate cyclase assay was performed. The protein concentration of the preincubation mixture was ≈690 μg/ml, and each assay was performed with ≈15 μg of protein. Results from three experiments (mean ± standard error) are shown for the percentage of stimulation induced by 5-HT. Each condition was assayed in triplicate in the presence or absence of 5-HT (10 μM). *, Paired t test with Bonferroni correction. *N.S.*, not significant.

Attempts to measure intracellular calcium flux as an indicator of phospholipase C activation. SM cells treated with serotonin (1 μM and 10 μM) after fura-2 loading demonstrated no change in the fluorescence emission, monitored at 510 nm after rapid alternating stimulation at 340 and 380 nm, thus indicating no significant change in calcium concentration. Treatment of the SM cells with the calcium ionophore, ionomycin (1 μM), however, did result in a measurable change in the fluorescence emission. Therefore, these data, in concert with the lack of inositol phosphate turnover after 5-HT stimulation, indicate that this receptor couples predominantly to adenylate cyclase in SM cells.

Discussion

Positive coupling between serotonin and adenylate cyclase has been described for more than three decades (35). Until recently, however, this 5-HT-mediated signal transduction pathway has lacked definitive receptor subtypes. Within the last 3 years a 5-HT receptor tied to activation of adenylate cyclase has been well characterized and labeled the 5-HT_4 receptor. Our data indicate that SM cells express a receptor with very similar characteristics. Known agonists for the 5-HT_4 receptor subtype act on SM cells to initiate the cascade of reactions that result in cAMP accumulation.

Shenker *et al.* (36) demonstrated serotonin stimulation of adenylate cyclase in adult guinea pig hippocampus. 5-HT displayed moderate affinity for its putative receptor in this tissue, ~100 nM. Subsequent studies by Shenker *et al.* (37, 38) and Markstein *et al.* (39) described the involvement of 5-HT_{1A} receptors stimulating cAMP accumulation in guinea pig and rat hippocampus, respectively. Before those reports, 5-HT_{1A} receptors had been solely linked to the inhibition of adenylate cyclase. Both groups documented a pharmacologic profile at

the receptor site consistent with a 5-HT_{1A} receptor, but the intriguing finding uncovered by Shenker *et al.* (37, 38) was a second lower affinity site for 5-CT-mediated stimulation of adenylate cyclase, a receptor site subsequently termed R_L by the authors. Interestingly, spiperone, a butyrophenone with high affinity for 5-HT_{1A} receptors, had a dramatically lower affinity for the R_L site, with a K_B of > 10 μM. This further suggested the possibility that a second 5-HT receptor subtype, in this instance the R_L site, could stimulate adenylate cyclase.

These interesting results supported the categorization of a novel 5-HT receptor suggested by Dumuis *et al.* (10) in 1989. They described a 5-HT receptor in mouse embryonic colliculi neurons that was remarkable for 1) a signal transduction pathway leading to the stimulation of adenylate cyclase, 2) a pharmacology highlighted by the moderate affinity of 5-HT (110 nM) and blockade of 5-HT-stimulated adenylate cyclase by ICS 205–930, and 3) a rank order of agonist potency at this receptor that placed 5-MT equipotent to 5-HT, whereas both were more potent than 5-CT.

These data again strongly pointed to a 5-HT receptor subtype different from the 5-HT_{1A} receptor subtype that was capable of stimulating adenylate cyclase. Dumuis *et al.* (10) reviewed the low affinity 5-CT site described by Shenker *et al.* (37, 38), as well as the other characteristics of the R_L receptor, and concluded that the similarities between those findings and the 5-HT receptor present in mouse embryonic colliculi neurons represented evidence for a new class of 5-HT receptors, 5-HT_4 receptors.

We examined SM cells for the presence of such a receptor primarily because Lee *et al.* (19) have shown that bovine pulmonary artery SM cells in primary culture respond to 5-HT, at least in part, with an increase in cAMP production. Our data support the conclusion that 5-HT and other agonists activate adenylate cyclase in these cells via a specific G protein-coupled receptor. Additionally, the 5-HT receptor in SM cells is linked primarily to the activation of adenylate cyclase and is not involved in other classical signal transduction pathways associated with 5-HT receptors, i.e., inositol phosphate turnover and phospholipase C activation.

Lee and Fanburg (20) have postulated a possible intracellular role for 5-HT as well as the involvement of a possible 5-HT surface receptor in triggering or maintaining second messenger pathways in SM cells. We felt that the binding of 5-HT to its receptor was likely to cause a membrane-delimited stimulation of cAMP accumulation. If so, the pathway for receptor-mediated activation of adenylate cyclase in SM cells probably was localized to the plasma membrane, where the cellular components necessary for this process, i.e., stimulatory G proteins and adenylate cyclase, are known to be present. Therefore, we pursued a membrane adenylate cyclase assay in an effort to distinguish the predominant site of 5-HT stimulation of adenylate cyclase. Our results effectively demonstrated that 5-HT stimulates adenylate cyclase through a membrane-associated pathway in SM cells. 5-HT mediated an increase in cAMP production in membrane particle preparations, and this effect could be competitively antagonized by the 5-HT receptor antagonist ICS 205–930. Moreover, 5-HT stimulated adenylate cyclase via a stimulatory G protein. Antiserum to the carboxyl terminus of the α subunit of G_s (RM-1), known to interfere with receptor-G protein coupling (34), markedly diminished cAMP generation in SM cells upon stimulation with 5-HT. This confirmed the presence of a stimulatory G protein linked to the 5-HT receptor in SM cells.

Pharmacologic analysis of the 5-HT receptor in SM cells remained necessary to classify this receptor as a 5-HT_4 receptor, as a 5-HT_4-like receptor, as a 5-HT_{1A} receptor, or as a novel, heretofore undescribed, receptor type. To accomplish this, we evaluated a number of compounds classically associated with serotonin receptors. We compared their documented potencies and binding affinities at other 5-HT receptors with the results obtained with cAMP RIA after exposure of SM cells to the compounds.

As previously noted, Dumuis *et al.* (10, 33) investigated the pharmacology of the 5-HT_4 receptor in fetal mouse colliculi and concluded that 5-HT and 5-MT were equipotent agonists and more potent than 5-CT. They also showed inhibition of 5-HT-stimulated adenylate cyclase by ICS 205–930 and MDL72222, but these two agents had lower affinities for the 5-HT_4 receptor than their documented affinities for 5-HT_3 receptors. Additionally, ICS 205–930 was more efficacious antagonizing the 5-HT response than was MDL 72222. Their group then observed that substituted benzamide derivatives that increased guinea pig ileal SM contraction at a putative 5-HT_4 receptor site (40) had a direct agonist effect on a 5-HT_4 receptor in hippocampal membrane preparations taken from these animals (29, 41). Compounds such as metoclopramide, cisapride, and zacopride stimulated the 5-HT_4 receptor in guinea pig hippocampus, leading to cAMP accumulation. These results enhanced the already unique pharmacology of the 5-HT_4 receptor.

Other 5-HT receptors that stimulate adenylate cyclase have been recently described as well. The 5-HT-*dro* receptor, a 5-HT receptor present in *Drosophila melanogaster*, definitely mediates 5-HT-stimulated cAMP production (42), with 5-HT being a more potent agonist than 5-MT. The pharmacology of this receptor, however, is less well defined and, therefore, is difficult to compare with our own data and the data derived by Dumuis *et al.* (10).

A 5-HT receptor stimulating adenylate cyclase in neuroblastoma NCB.20 cells has also been characterized by Conner and Mansour (28). This 5-HT_4-like receptor manifested potencies for 5-HT and 5-MT similar those of the receptor described by Dumuis *et al.* and displayed a rank order of agonist potency that correlated with that reported for a 5-HT_4 receptor (5-HT = 5-MT $\gg$ 5-CT). The 5-HT receptor in NCB.20 cells, however, differed somewhat from the receptor present in murine embryonic colliculi neurons, in two respects. First, 5-CT was a less efficacious agent, compared with 5-HT, in stimulating adenylate cyclase in NCB.20 cells. These two compounds were equally efficacious in mouse embryonic colliculi neurons. Second, MDL 72222, a weak antagonist of the 5-HT_4 receptor reported by Dumuis *et al.* (10, 33), demonstrated no significant inhibition of 5-HT-stimulated adenylate cyclase activity in NCB.20 cells (28).

Thus, only a few uniform characteristics define the pharmacology of 5-HT_4-like receptors at present, i.e., 1) 5-HT binds to the receptor with an affinity approximating 100 nM and 2) other distinguishing tryptamine derivatives yield a rank order of potency such that 5-HT and 5-MT are equipotent and both are far more potent than 5-CT. These two features clearly differentiate 5-HT_4 and 5-HT_4-like receptors from 5-HT_1 receptors, where 5-CT $\geq$ 5-HT $>$ 5-MT, and 5-HT_2 receptors,

where none of these compounds have an affinity greater than 1 μM.

Characteristics that categorize antagonists at this receptor site are suggested from findings described by Dumuis *et al.* (10, 33) documenting ICS 205–930 as a more potent antagonist than MDL 72222 at the 5-HT_4 receptor site in embryonic mouse colliculi. Conner and Mansour (28) also demonstrated at the 5-HT_4-like receptor in NCB.20 cells that mianserin was more potent than ketanserin as an antagonist of 5-HT-mediated cAMP accumulation. Both of these results clearly highlight the distinct nature of the 5-HT_4-like receptors, because ICS 205–930 and MDL 72222 are essentially equipotent at 5-HT_3 receptor sites, whereas mianserin and ketanserin display a reversal of antagonist potencies and much higher affinities at 5-HT_2 receptor sites, compared with 5-HT_4 receptors.

Our data delineate some of the characteristics of the SM cell 5-HT receptor. The nature of the 5-HT response in SM cells effectively excludes the involvement of a 5-HT_2 or 5-HT_3 receptor in the activation of adenylate cyclase. As previously mentioned, the potency of mianserin relative to that of ketanserin, their respective affinities for the 5-HT receptor in SM cells, the lack of measurable inositol phosphate turnover or phospholipase C activation, and, finally, the lack of receptor stimulation with DOI, a relatively specific 5-HT_2 and 5-HT_{1C} receptor agonist, clearly establish that the SM cell receptor is not a 5-HT_2 or a 5-HT_{1C} receptor. The differential potencies of ICS 205–930 and MDL 72222, the integral involvement of a stimulatory G protein in the signal transduction pathway, and the lack of any inhibitory effect mediated by metoclopramide all distinguish the SM cell 5-HT receptor from the 5-HT_3 receptor subtype.

Additionally, the SM cell receptor is clearly different from a 5-HT_{1B}, 5-HT_{1C}, or 5-HT_{1D} receptor. First, the signal transduced by the SM cell receptor is stimulation of adenylate cyclase. This obviously contrasts with the inhibition of adenylate cyclase mediated by 5-HT_{1B} and 5-HT_{1D} receptors (43, 44) and the activation of phospholipase C mediated by 5-HT_{1C} receptors (5). Second, specific pharmacologic agents that bind preferentially to these receptor types, including DOI as noted above and TFMPP, an agent with moderate to high affinity for 5-HT_{1B} receptors, showed no significant stimulation of the SM cell 5-HT receptor.

The chief characteristics of the SM cell 5-HT receptor suggested that it was either a 5-HT_{1A} or a 5-HT_4-like receptor. The possible coexistence of 5-HT_{1A} and 5-HT_4-like receptors in SM cells also confounded an immediate identification of the SM cell receptor type.[2] On the basis of several distinct pharmacologic findings, however, the SM cell 5-HT receptor linked to increased cAMP accumulation is of a 5-HT_4-like subtype. The agonist potency of 5-HT at the SM cell receptor is approximately 100 nM. The rank order of agonists, 5-HT = 5-MT $\gg$ 5-CT, and antagonists, ICS 205–930 > MDL 72222 and mianserin > ketanserin, is wholly consistent with a 5-HT_4-like receptor. Additionally, the approximate pA_2 of 6.3 for ICS 205–930 correlates remarkably well with previous reports regarding this antagonist at 5-HT_4 receptors (40). Finally, adenylate cyclase stimulation mediated by 8-OH-DPAT was antagonized by ICS 205–930, whereas NAN-190, a specific 5-HT_{1A} antagonist, had no inhibitory effect at all on 8-OH-DPAT-stimulated cAMP production in SM cells.

Having established the presence of a 5-HT_4-like receptor in SM cells, we compared our findings with those previously reported for 5-HT_4 receptors in the central nervous system. Only those compounds with detectable effects resulting in stimulation of adenylate cyclase activity or inhibition of serotonin-stimulated adenylate cyclase activity are shown in Fig. 6. 8-OH-DPAT was excluded from the analysis because of its reported lack of effect at these receptor sites, as well as to illustrate the distinct nature of the 5-HT receptors in NCB.20 cells and mouse embryonic colliculi neurons. Fig. 6, A and B, shows a poor correlation between agonist and antagonist potencies in the human neuroblastoma NCB.20 cell line and the 5-HT receptor in SM cells (r^2 = 0.334 and r^2 = 0.167, respectively). Conversely, a much stronger correlation is evident in Fig. 6C, which compares the 5-HT agonist potencies in mouse embryonic colliculi neurons and SM cells (r^2 = 0.918). A comparison between the antagonist affinities at the fetal mouse

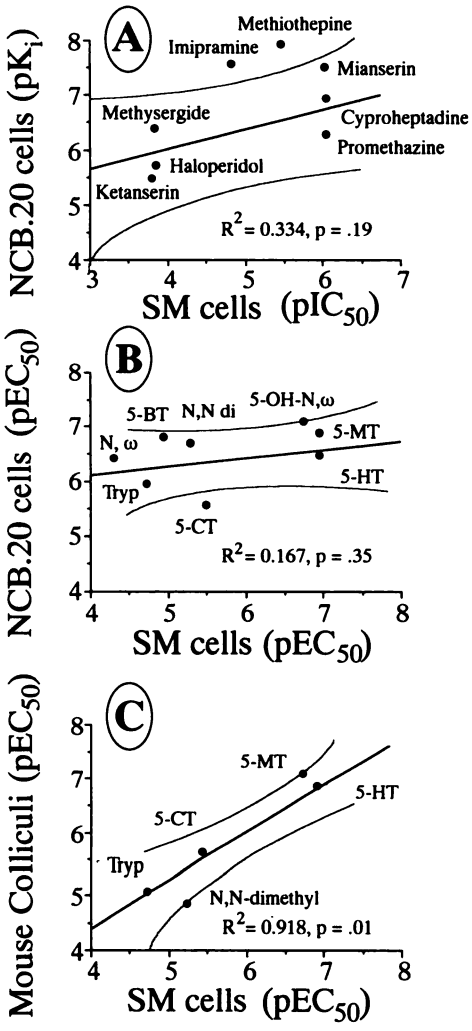

Fig. 6. Correlation between potencies of serotonin agonists and antagonists for the 5-HT_4-like receptor in NCB.20 cells, the 5-HT_4 receptor in fetal mouse colliculi, and the 5-HT receptor in SM cells. Affinities (K_i) and EC_{50} values were obtained from data published by Conner and Mansour (28) and Dumuis *et al.* (10). For the NCB.20 neuroblastoma 5-HT receptor, correlation between antagonist potencies is presented in A and correlation between agonist potencies in B. For the mouse embryonic colliculi 5-HT_4 receptor, correlation between agonist potencies is shown in C. Correlation plots were determined by nonweighted linear regression as described in Materials and Methods. *Curved lines*, 95% confidence intervals for the *y* values.

colliculi receptor and the SM cell receptor is hampered by the fact that Dumuis *et al.* (10, 33) documented only two compounds that effectively inhibited the 5-HT response, ICS 205–930 and MDL 72222. These two antagonists retain the same relative potency at both the embryonic mouse colliculi and SM cell receptor sites, i.e., ICS 205–930 > MDL 72222. These consistent differences between ICS 205–930 and MDL 72222, the discrepant pharmacology noted in Fig. 6, A and B, and the findings displayed in Fig. 6C clearly demonstrate that the 5-HT_4-like receptor in SM cells is much more closely related to the 5-HT_4 receptor in fetal mouse colliculi than the 5-HT_4-like receptor in NCB.20 cells. An additional and unique feature of the SM cell 5-HT receptor is the potency of 8-OH-DPAT at the receptor site. This property has not been documented at other 5-HT_4 and 5-HT_4-like receptors (10, 28). These findings strongly suggest that heterogeneity exists among 5-HT_4-like receptors, and there appear to be sufficient differences between receptor subtypes to argue for a 5-HT_4-like receptor "family."

Possible roles for this receptor in pulmonary artery SM cells have already been postulated. Lee *et al.* (19, 20, 45) have linked serotonin to mitogenesis and configurational change in this specific cell line. The 5-HT_4-like receptor in these cells may, therefore, be directly involved in these cellular changes. As previously mentioned, several investigators, including Elswood *et al.* (46), have identified a putative 5-HT_4 receptor involved in concentration-related contraction of isolated guinea pig ascending colon. Thus, the 5-HT_4 receptor may well play an important physiologic role in contraction and relaxation responses of SM cells.

To summarize, our data indicate that a 5-HT_4-like receptor is present in bovine pulmonary artery SM cells. This receptor, like other 5-HT_4 receptors described, stimulates cAMP accumulation. The mode of activation of adenylate cyclase is via the α subunit of G_s. The SM cell receptor agonist profile bears characteristics notably far more similar to those of the receptor in mouse embryonic colliculi than to those of the 5-HT receptor in neuroblastoma NCB.20 cells. The only great disparity is the high apparent affinity of the SM cell 5-HT receptor for 8-OH-DPAT. Our data thus call into question the selective affinity of 8-OH-DPAT for 5-HT_{1A} receptor sites. This compound acted at the 5-HT_4-like receptor in SM cells to stimulate cAMP production in the presence of a 5-HT_{1A} antagonist, NAN-190. This suggests that distinct receptor entities within the recently defined 5-HT_4 class of serotonin receptors exist and 8-OH-DPAT appears to be a useful discriminatory compound for evaluating 5-HT receptors tied to adenylate cyclase stimulation.

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