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A Nonclassical 5-Hydroxytryptamine Receptor Positively Coupled with Adenylate Cyclase in the Central Nervous System

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SUMMARY

A nonclassical 5-hydroxytryptamine (5-HT) receptor mediates the stimulation of adenylate cyclase activity in mouse embryo colliculi neurons in primary culture. The pharmacological profile characterized with agonists and antagonists suggests that this 5-HT receptor does not appear to correspond to a known 5-HT receptor. On this 5-HT receptor, 5-HT (EC_{50} = 109 ± 17 nM) and 5-methoxytryptamine (5-MeOT) were equipotent agonists. The other tryptamine derivatives, 5-carboxamidotryptamine (5-CT) and 5-methoxy-*N,N*-dimethyltryptamine (5-MeOT-*N,N*-DMT), were full potent agonists, whereas tryptamine, bufotenine, and 2-CH_3-5-HT were weak partial agonists. Two selective 5-HT_{1A} agonists: 8-hydroxy-2-(di-*n*-propylamino)-tetralin (8-OH-DPAT) and ipsapirone, could not stimulate adenylate cyclase. RU 24969, a tetrahydropyridoindole derivative that is a potent 5-HT_{1A} and 5-HT_{1B} agonist was also inactive, whereas RU 28253, another member of this series, could stimulate cAMP production. The action of antagonists acting on 5-HT_1 or 5-HT_2 receptors, such as methiothepin (5-HT_1 and 5-HT_2), metergoline (5-HT_1 and 5-HT_2), spiperone (5-HT_{1A} and 5-HT_2), (−)-pindolol (5-HT_{1B}), mesulergine (5-HT_{1C}), and ketanserin (5-HT_2), were almost inactive in reversing the 5-HT stimulating effect. The selective 5-HT_3 antagonist ICS 205 930 was a full competitive antagonist at this receptor. Nevertheless, MDL 72222, which is also a 5-HT_3 antagonist, was very weak in antagonizing the 5-HT stimulatory effect. A receptor with similar characteristics has also been found in guinea pig hippocampal membranes. In these membranes, the second receptor of low affinity for 5-HT, termed R_L, which is positively coupled to adenylate cyclase, was also antagonized by ICS 205 930. The relatively low affinity of this hippocampal receptor for 5-CT, its stimulation by RU 28253 but not by RU 24969, and its previously reported pharmacological characteristics support the contention that this 5-HT receptor and the 5-HT receptor of mouse embryo colliculi neurons in primary culture (both positively coupled to cAMP formation) present great homologies. Inasmuch as none of the classical specific 5-HT_1 and 5-HT_2 agonists or antagonists interact with these 5-HT receptors, it is unlikely that they belong to 5-HT_1 or 5-HT_2 receptor categories. Furthermore, inasmuch as only one 5-HT_3 antagonist (ICS 205 930) blocks their activity and another 5-HT_3 agonist (MDL 72222), as well as the specific 5-HT_3 agonist 2-CH_3-5-HT, were almost inactive, the possibility that they belong to the 5-HT_3 category is excluded. We propose that the 5-HT receptor of mouse embryo colliculi neurons, as well as the low affinity 5-HT receptor (R_L) of the guinea pig hippocampus, belong to a new category of 5-HT receptors that we suggest calling 5-HT_4.

With the recent discovery of a variety of drugs selectively acting at 5-HT receptors, two main categories of 5-HT binding sites (5-HT_1 and 5-HT_2) have been described in the central nervous system (1, 2).

Subsequent analysis of the 5-HT_1 sites based on radioligand binding studies revealed four distinct subtypes, each with distinctive pharmacology and termed 5-HT_{1A} (2–5), 5-HT_{1B} (6, 7), 5-HT_{1C} (8, 9), and 5-HT_{1D} (10). In contrast, 5-HT_2 receptors, initially called D receptors (11), appear to be homogeneous (12) at both the peripheral and central nervous systems.

A third type of receptor, recently termed 5-HT_3, which probably corresponds to the 5-HT M receptors described 30 years ago (11), have clearly been identified in the peripheral nervous system by functional experiments (13–18). Considerable data support that 5-HT_3 receptors are involved in depolarization of peripheral neurons (17), in contraction of vascular smooth muscles (19, 20), and in increasing neurotransmitter release (13, 17, 20). Recently, results suggest that cisplatine-induced emesis could be blocked by 5-HT_3 receptor antagonists (21).

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; 5-CT, 5-carboxamidotryptamine; bufotenine, 5-hydroxy-*N,N*-dimethyltryptamine; 5-MeOT, 5-methoxytryptamine; 5-MeO-*N,N*-DMT, 5-methoxy-*N,N*-di-methyltryptamine; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin; ipsapirone (TVXQ 7821),2-(4-4-2-pyramidinyl, 1,2-benzoisothiazol-3-(2*H*)-one-1,1 dioxide hydrochloride; *d*-LSD,*d*-lysergic acid diethylamide; *E*, efficacy; EC_{50}, half maximal efficacy; PCPA, parachlorophenylalanine; ICS 205 930, (3α-tropanyl)-1*H*-indole-3-carboxylic acid ester; MDL 72222, 1α*H*,3α,5α*H*-tropan-3-yl-3,5-dichlorobenzoate; RU 24969, 5-methoxy-3-(1,2,3,6-tetrahydropyridine-4-yl)indol; RU 28253, 5-methoxy-3-(1,2,5,6-tetrahydropyridine-4-yl)indol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

The first experimental evidence for the existence of 5-HT_3 receptor binding in mammalian brain was provided by Kilpatrick *et al.* (22) using [^{3}H]GR65630, a selective 5-HT_3 antagonist in rat brain membranes. The binding of [^{3}H]ICS 205 930, a potent selective 5-HT_3 antagonist (13) has also been reported in NG 108-15 neuroblastoma glioma cells (23). Finally, [^{3}H] quipazine labeled 5-HT_3 receptors in rat brain cortical membranes (24). The recent development of potent selective 5-HT_3 receptor antagonists, such as MDL 72222 (14), ICS 205 930 (13), BRL 24924 (15), BRL 43694 (16), metoclopramide (17), and GR 38032F (18), have provided clear evidence that the functional responses described above are mediated by 5-HT_3 receptors. Nevertheless, no biochemical responses have yet been correlated with 5-HT_3 receptors, as opposed to 5-HT_1 and 5-HT_2 receptors.

It is now established that three 5-HT_1 receptors of the four identified subtypes modulate cAMP levels. In hippocampal membranes of rat and guinea pig and in neurons in primary culture, it was clearly shown that 5-HT_{1A} receptors mediate both inhibition of stimulated adenylate cyclase (25–28) and stimulation of adenylate cyclase under basal conditions (29–31). A recent study (32) gives evidence that 5-HT_{1B} receptors of rat substantia nigra are also negatively coupled to an adenylate cyclase. 5-HT_{1D} sites showing low affinity for all 5-HT_{1A}-, 5-HT_{1B}-, and 5-HT_{1C}-selective drugs (10) seem to be identified only in brains devoid of 5-HT_{1B} sites, such as bovine, pig, and human brains (10, 33). Like 5-HT_{1B} sites, 5-HT_{1D} sites are negatively coupled to adenylate cyclase (34).

5-HT_{1C} sites (8, 35) are specifically concentrated in choroid plexus and cortex of various species. They appear to be coupled to a phospholipase C to trigger the inositol triphosphate-diacylglycerol system (36). A similar coupling has been established for the mechanism of action of 5-HT_2 receptors, both at the periphery and in the central nervous system (37–41).

The major subtypes of 5-HT sites have therefore been well characterized for their effects on second messenger systems. Nevertheless, Shenker *et al.* (31) reported that guinea pig hippocampal membranes contained two distinct 5-HT receptors positively coupled to adenylate cyclase, a receptor called R_H (high affinity for 5-HT), which is a functional correlate of the 5-HT_{1A} binding site, and a second one, R_L (low affinity for 5-HT), having a different pharmacology from that of 5-HT_1 receptors.

We have recently reported a preliminary study showing that colliculi neurons from fetal mice in primary culture contain a 5-HT receptor positively coupled to adenylate cyclase, with pharmacological characteristics distinct from those of 5-HT_1 receptors (42). Additional work presented here on both systems mediating stimulation of adenylate cyclase, i.e., mouse embryo colliculi neurons in primary culture and guinea pig hippocampal membranes, suggests that the 5-HT receptors of mouse embryo colliculi neurons present some pharmacological homologies with the second 5-HT receptor in guinea pig hippocampal membranes (R_L), previously described by Shenker *et al.* (31). The pharmacological profile did not provide a way of classing these receptors in a known 5-HT_1 binding site subtype.

Materials and Methods

Culture of Mouse Embryo Colliculi Neurons

Neurons in primary culture were generated from colliculi of 14–15-day-old mouse embryos. They were grown for 6 days in serum-free medium supplemented with a prepared hormone mixture in 12-well Costar dishes (10^6 cells/well) at 37° in a humidified atmosphere (5% CO_2/95% air). These cultures were prepared as previously described for striatal and cortical neurons in a defined medium (43).

Cyclic AMP Formation

On the sixth day of culture and before each experiment, cells were incubated with 2 μCi/ml [^{3}H]adenine (24 μCi/mol; Amersham, UK) in a culture medium. After 2 hr, the cultures were washed and incubated with the medium containing 0.75 mM isobutylmethylxanthine, 0.1 μM forskolin, and test agents (all prepared in culture medium in a 1-ml volume). The reaction was stopped by aspiration of the media and addition of 1 ml of ice-cold 5% trichloracetic acid. Cells were scraped with the aid of a rubber policeman and 100 μl of 5 mM ATP and 5 mM cAMP were added to the mixture. Cellular proteins were centrifuged at 5000 × *g* and [^{3}H]ATP and [^{3}H]cAMP were separated by sequential chromatography on Dowex and alumina columns, as described by Salomon *et al.* (44). cAMP formation is expressed as per cent conversion of [^{3}H]ATP to [^{3}H]cAMP. We have previously shown that, in neuronal cultures, 0.1 μM forskolin does not modify basal cyclic AMP concentrations but increases neurotransmitter efficacy in cyclic AMP production, whereas potency is unaffected (see Fig. 1 in Ref. 45).

Adenylate Cyclase Assay

Male adult guinea pigs received a chronic drug treatment with PCPA (300 mg/kg/day). The animals were treated 3 days, 2 days, and the day before decapitation and the last dose was given 4 hr before decapitation. Hippocampi were removed and membranes were prepared as described by Bockaert *et al.* (27). Tissues were homogenized using a Dounce homogenizer (four strokes) in 2 mM Tris-maleate, pH 7.2, containing 2 mM EGTA and 300 mM sucrose at 4° (40 mg in 0.8 ml). Homogenates were then filtered through a silk screen (150 μm pore diameter).

The adenylate cyclase assay was performed in the absence of forskolin with hippocampal membranes as previously described (27). Incubation was carried out at 30°. The cAMP that formed during the reaction was extracted according to the method of Salomon *et al.* (44). Protein was determined according to the method of Lowry *et al.* (46).

Data Analysis

Experiments performed on mouse embryo colliculi neurons. In all the figures, the mean values of at least three experiments performed in duplicate have been given, ± standard errors, which are represented by vertical bars. EC_{50} refers to the agonist concentrations yielding 50% of the maximal activation determined directly on each concentration response curve. The K_i values of antagonists were calculated from the concentration of the drug reversing the stimulation obtained with 5-HT (1 μM) by 50%, using the Cheng-Prusoff equation (47) or by using the method of Arunlakshana and Schild (48). The equation used was $\log(dr - 1) = \log[B] - \log K_b$. dr = ratio of EC_{50} values of an agonist in the presence and absence of various concentrations of antagonists; $[B]$ = concentration of the antagonist; K_b = dissociation constant for the antagonist.

Experiments performed on hippocampal membranes. Computer analyses of the concentration-response curves were performed as previously described (49), assuming that there were only two 5-HT receptor subtypes. Minimization of the $\sum [Y_{exp} - Y_{theoretical}]^2$ was computerized by a nonlinear regression analysis (50).

Drugs

The following drugs were generously donated: 5-CT (P. P. A. Humphrey, Glaxo Group Research, Hertfordshire, UK), ipsapirone (TVXQ 7821) (J. Traber, Troponwerke GmbH and Co., Cologne, FRG), RU 28253 and RU 24969 (F. J. Pujol, Roussel-Uclaf, Romainville, France), metergoline and methysergide (H. Gozlan, Faculté de Médecine, Pitié-Salpêtrière, Paris, France), spiperone and ketanserin (J. Leysen, Janssen Pharmaceutica, Beerse, Belgium), *d*-LSD, mesulergine, (−)-pindolol, ICS 205 930, and 2-CH_3-5-HT (Sandoz Ltd., Basel, Switzerland),

methiothepin (Hoffman-La-Roche, Basel, Switzerland), and MDL 72222 (J. Fozard, Merrel Dow Research Institute, Strasbourg, France).

The purchased drugs were the following: 5-HT, 5-Me-OT, 5-Me-O-*N,N*-DMT, and tryptamine hydrochloride (Sigma Chemical Co., St. Louis, MO), bufotenine (Regis, Morton-Grove, IL), 8-OH-DPAT (Research Biochemical Inc., Wayland, MA), and 5-phenylbiguanide (Alkirch Chemi, Steinheim, FRG).

Results

Effect of 5-HT and specific 5-HT$_1$ agonists on cyclic AMP production in mouse embryo colliculi neurons in primary culture. Similar to its effects on adenylate cyclase in newborn rat colliculi homogenates (51), in striatal and cortical neurons (25), 5-HT stimulated cyclic AMP production in intact colliculi neurons in primary culture with an EC_{50} of 109 ± 17 nM (15 experiments).

In the absence of 5-HT, the per cent conversion was 1.1 ± 0.2% (15 experiments and, in the presence of 10^{-5}M 5-HT, the percent conversion was 2.8 ± 0.3% (15 experiments). The stimulation resulted in a 2.5- to 3-fold increase in the basal level of cAMP.

5-CT, a full potent specific 5-HT$_1$ agonist, was able to stimulate adenylate cyclase, eliciting 100% of the maximal activity of 5-HT at 3×10^{-5} M. The very low affinity of 5-CT (EC_{50} = 3160 ± 630 nM) (five experiments) suggested that the 5-CT stimulation was not mediated by a 5-HT$_1$ receptor. The 5-CT concentration-response curve (Fig. 1) was monophasic, as compared with those obtained with guinea pig hippocampal membranes, which were distinctly biphasic as shown in Fig. 5A and previously by Shenker *et al.* (31).

8-OH-DPAT and ipsapirone, two selective 5-HT$_{1A}$ agonists, as well as RU 24969, a potent 5-HT$_{1A}$ and 5-HT$_{1B}$ agonist, failed to stimulate cAMP production. Only one tetrahydropyridoindol derivative, RU 28253, was able to stimulate adenylate cyclase, with mean EC_{50} values of 560 ± 120 nM (three experiments) (Fig. 1; Table 1).

Effect of tryptamine derivatives on cyclic AMP production. As reported in Fig. 2 and Table 1, 5-MeOT was as potent as 5-HT (EC_{50} = 100 ± 12 nM, four experiments). 5-MeO-*N,N*-DMT elicited 100% of the maximal activity obtained with 5-HT but had a 17-fold lower affinity (EC_{50} = 17780 ± 3800 nM, three experiments). Bufotenine was a weak partial agonist (EC_{50} = 1580 ± 350 nM, three experiments), eliciting 64% of the maximal 5-HT effect. Tryptamine and 2-CH_3-5-HT (a highly selective 5-HT$_3$ agonist) were very weak in mimicking the 5-HT stimulation. Only 50% of the maximal 5-HT effect was reached with these two drugs at 100 μM.

Effect of specific 5-HT$_1$, 5-HT$_2$ antagonists on 5-HT stimulation of cAMP in mouse embryo colliculi neurons. Methiothepin and metergoline, which are potent 5-HT$_1$ and 5-HT$_2$ antagonists, failed to reverse cAMP stimulation induced by 1 μM 5-HT. Spiperone, a rather potent selective 5-HT$_{1A}$ and 5-HT$_2$ antagonist was also inactive (Table 2).

Increasing concentrations of ketanserin, a highly selective 5-HT$_2$ antagonist (12), mesulergine, a rather selective 5-HT$_{1C}$ antagonist (8), and (−)-pindolol, a good 5-HT$_{1B}$ antagonist, had no effect on 5-HT-stimulated adenylate cyclase activity (Table 2).

Effect of 5-HT$_3$ antagonists. Two selective 5-HT$_3$ receptor antagonists were tested in order to complete the pharmacological profile of the 5-HT receptor coupled with an adenylate cyclase in mouse embryo colliculi neurons. ICS 205 930, a highly selective and potent 5-HT$_3$ antagonist (13), inhibited the effect produced by 1 μM 5-HT up to 100%, as reported in Fig. 3. The ability of three concentrations of ICS 205 930 (10, 30, and 100 μM) to shift the 5-HT concentration response curve to the right was tested (Fig. 4A). The three concentration-response curves showed that ICS 205 930 competitively inhibited the effect of 5-HT without depressing the E_{max}. This was confirmed by a Schild plot (slope 1.2 ± 0.1, three experiments). The K_i value for ICS 205 930 was 997 ± 353 nM (six experiments). Another selective 5-HT$_3$ antagonist, MDL 72222 (14), was rather weak. It reversed 50% of the stimulatory effect of 5-HT at a high concentration (10^{-4} M) (Fig. 3).

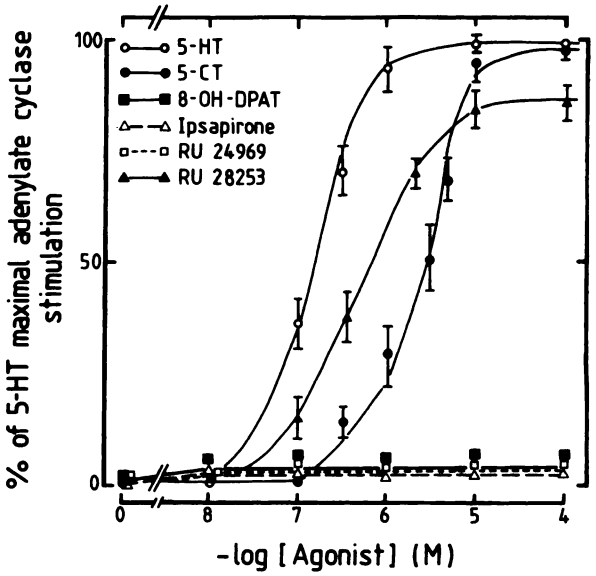

Fig. 1. Effect of various concentrations of several 5-HT$_1$ agonists, 5-HT, 5-CT, 8-OH-DPAT, ipsapirone, RU 24969, and RU 28253, on cAMP production in colliculi neurons in primary culture. Cells were incubated at a low concentration of forskolin (0.1 μM) and increasing concentrations of each agonist. Conversion of [^{3}H]ATP to [^{3}H]cAMP was determined after 5 min at 37°. In the absence of 5-HT, the per cent conversion was 1.1 ± 0.2% and 2.8 ± 0.3% in the presence of 10^{-5} M 5-HT (15 experiments). These results are expressed as the percentage of the maximal stimulatory effect of 5-HT. The values are the means ± standard errors of at least three separate experiments each performed in duplicate.

Presence of a 5-HT-stimulated adenylate cyclase inhibited by ICS 205 930 in adult guinea pig hippocampal membranes. Shenker *et al.* (31) reported that two 5-HT receptors were able to stimulate adenylate cyclase in guinea pig hippocampus, one with a high affinity for 5-HT (R_H), characterized as being a 5-HT$_{1A}$ receptor, and the other with a low affinity for 5-HT (R_L), the pharmacology of which is not classical. 5-CT was better than 5-HT in clearly distinguishing between these two receptors, because a clear biphasic dose-activation curve was obtained with this agonist. We confirmed such a biphasic dose-activation curve for 5-CT (Fig. 5A). The first component of the 5-CT curve, which reached a plateau over the concentration range of 0.3 to 1 μM, yielded an EC_{50} equal to 13.1 ± 2.2 nM three experiments). At higher concentrations, 5-CT elicited a further increase in adenylate cyclase activity (EC_{50} = 29500 ± 3200 nM, three experiments). The E_{max} at the first plateau was equal to about 38 ± 5% (three experiments) of the maximal response to 5-CT.

We also found that the 5-HT dose-activation curves can be fitted to a model describing the action of an agonist on two

TABLE 1

Activity of agonists at 5-HT receptors positively coupled to adenylate cyclase in mouse embryo colliculi neurons in primary culture and at 5-HT receptors of low affinity (R_L) in guinea pig hippocampal membranes

The effects of agonists on adenylate cyclase activity were determined as described under Materials and Methods. Data are expressed as EC_{50}, means ± standard error of at least three separate experiments. The efficacy (E_{max}) of the agonists is the maximal stimulating effect of adenylate cyclase as a percentage of the maximal stimulatory effect of 5-HT. A comparison with EC_{50} values at 5-HT receptors of low affinity (R_L) in guinea pig hippocampal membranes taken from Shenker *et al.* (31) was made (data reported in the *right column*). EC_{50} values were calculated as described under Materials and Methods.

Agonists	Colliculi neurons		Hippocampal membranes		Drug Selectivity
	EC_{50}	E_{max}	RL EC_{50}[a]	RL EC_{50}[b]	
	nM	%	nM		
5-MeOT	100 ± 12	100		578 ± 107	5-HT_{1A} + 5-HT_{1B} + 5-HT_{1D} agonist
5-HT	109 ± 17	100	263 ± 62	414 ± 53	5-HT agonist
RU 28253	560 ± 120	85	3500 ± 350		5-HT_{1A} + 5-HT_{1B} agonist
Bufotenine	1580 ± 350	64		4790 ± 1640	5-HT_{1A} + 5-HT_{1B} + 5-HT_{1D} agonist
5-CT	3160 ± 630	100	29500 ± 3200	31300 ± 4500	5-HT_{1A} + 5-HT_{1B} + 5-HT_{1D} agonist
5-MeO-*N*,*N*-DMT	17780 ± 3800	100			5-HT_{1A} + 5-HT_{1B} + 5-HT_{1D} agonist
Tryptamine	>10000	50		33100 ± 9600	Nonselective 5-HT agonist
d-LSD	>10000	<50			Nonselective 5-HT_1 agonist
2-CH_3-5-HT	>10000	<50	Inactive		5-HT_3 agonist
8-OH-DPAT	Inactive		Inactive	>50000	5-HT_{1A} agonist
Ipsapirone	Inactive				5-HT_{1A} agonist/antagonist
Buspirone	Inactive				5-HT_{1A} agonist/antagonist
Metergoline	Inactive				5-HT_{1A} agonist/5-HT_1 antagonist + 5-HT_2 antagonist
Methysergide	Inactive				5-HT_{1A} agonist/5-HT_1 antagonist + 5-HT_2 antagonist
1-Phenylbiguanide	Inactive			Inactive	5-HT_3 agonist
RU 24969	Inactive		Inactive		5-HT_{1A} + 5-HT_{1B} + 5-HT_{1D} agonist

[a] EC_{50} values takena from our own experiments.
[b] EC_{50} values taken from experiments by Shenker *et al.* (31).

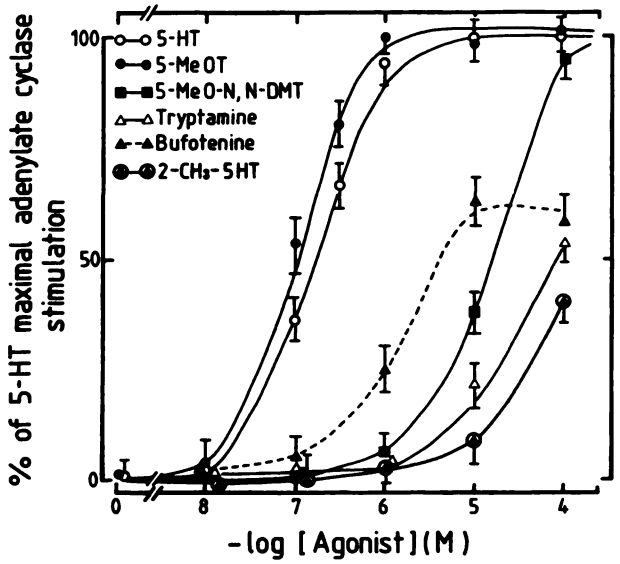

Fig. 2. Stimulation of cAMP formation by six tryptamine derivative agonists, 5-HT, 5-MeOT,5-MeO-*N*,*N*-DMT,bufotenine,tryptamine, and 2-CH_3-5-HT. Cells were incubated at a low concentration of forskolin (0.1 μM) and increasing concentrations of each agonist. Conversion of [^{3}H]ATP to [^{3}H]cAMP was determined after 5 min at 37°. In the absence of 5-HT, the per cent conversion was 1.1 ± 0.2% and 2.8 ± 0.3% in the presence of 10^{-5} M 5-HT (15 experiments). These results are expressed as the percentage of the maximal stimulatory effect of 5-HT. Data are the mean of three to five separate experiments each performed with triplicate determinations. The variations of the data points are indicated by the *bars*, for each concentration drug.

independent receptors (49). The E_{max} of the high affinity 5-HT receptor (R_H) and of the low affinity 5-HT receptor were 46 ± 4% and 54 ± 4%, respectively (three experiments), whereas the EC_{50} values were 23 ± 4 mM (three experiments) and 263 ± 62 nM (three experiments), respectively.

As observed in mouse embryo colliculi neurons, RU 28253 was able to stimulate the 5-HT receptors positively coupled with cyclic AMP production in hippocampal membranes. The dose-activation curves were also fitted to a model describing the actions of this agonist or two receptors. The EC_{50} values were 25 ± 5 nM and 3500 ± 350 nM (three experiments) (Fig. 5A; Table 1).

The adenylate cyclase stimulation obtained with low concentrations of 5-CT was inhibited by low spiperone concentrations (K_i = 4.6 ± 1 nM, three experiments) (data not shown). This confirms that the first component of the 5-CT dose-response curve (R_H) was due to the stimulation of 5-HT_{1A} receptors, as previously reported by Shenker *et al.* (31). As also described by Shenker *et al.* (31), we found that spiperone was very weak in inhibiting the low affinity component (R_L) of the 5-HT dose-response curve (K_i > 10000 nM) (Table 2). The efficacy of RU 24929 in stimulating the adenylate cyclase was only 35% of that for 5-HT (data not shown). This effect was blocked by low spiperone concentrations (K_i = 8 nM), suggesting that this compound stimulates R_H and not R_L (Table 1).

In order to determine whether this low affinity component of the 5-CT dose-response curve presents other similarities to the 5-HT induced response in mouse embryo colliculi neurons, we tested the effect of ICS 205 930. Indeed, ICS 205 930 inhibited the low affinity component of the 5-CT dose-response curve (100 μM 5-CT) with a K_i equal to 454 ± 120 nM (Fig. 5C; Table 2). As reported by Shenker *et al.* (31), MDL 72222 was completely inactive (Fig. 5C). In contrast, the high affinity component of the 5-CT dose-response curve (0.3 μM 5-CT) was insensitive to both ICS 205 930 and MDL 72222 (Fig. 5B). The absence of effect of ICS 205 930 on the high affinity component of the 5-CT dose-response curve (Fig. 5B) explains why ICS 205 930 inhibited only by 65 ± 10% (three experiments) the adenylate cyclase stimulated by 100 μM 5-CT (Fig. 5C).

Discussion

The concept of multiple receptors for a given neurotransmitter has now been demonstrated for the great majority of sys-

TABLE 2

Activity of a series of 5-HT antagonists on 5-HT receptors in reversing the stimulation of adenylate cyclase obtained with 5-HT in mouse embryo colliculi neurons in primary culture on 5-HT receptors of low affinity in guinea pig hippocampal membranes

The effect of antagonists were determined as described under Materials and Methods. Data are expressed as K_i, means ± standard error of at least three separate experiments. The K_i values of antagonists were calculated from the concentration of the drug reversing by 50% the stimulation obtained with 5-HT (1 μM) in mouse embryo colliculi neurons and with 5-CT (100 μM) in guinea pig hippocampal membranes, as described under Materials and Methods. A comparison with the K_i values at 5-HT (R_L) receptors in guinea pig hippocampal membranes taken from Shenker *et al.* (31) was made (values reported in the *right column*).

Antagonists	Colliculi neurons K_i	Hippocampal membranes		Drug Selectivity
		RL K_i[a]	RL K_i[b]	
		nM		
Spiperone	Inactive	>10000	≥3000	5-HT_{1A} + 5-HT_2 antagonist
Metergoline	Inactive			5-HT_1 + 5-HT_2 antagonist
Methiothepin	Inactive			5-HT_1 + 5-HT_2 antagonist
Mesulergine	Inactive			5-HT_{1C} antagonist
Ketanserine	Inactive		>100	5-HT_2 antagonist
(−)-Pindolol	Inactive			5-HT_{1A} + 5-HT_{1B} antagonist
ICS 205 930	997 ± 353	454 ± 120		5-HT_3 antagonist
MDL 72222	>5000	Inactive	Inactive	5-HT_3 antagonist
Cocaine	Inactive		Inactive	5-HT_3 antagonist

[a] EC_{50} values taken from our own experiments.
[b] EC_{50} values taken from experiments by Shenker et al. (31).

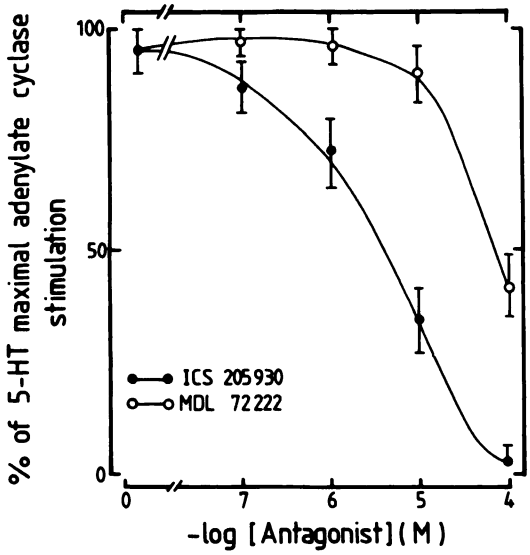

Fig. 3. Effect of highly selective 5-HT_3 antagonists on 5-HT stimulation of cAMP formation in colliculi neurons. In the presence of 0.1 μM forskolin and 1 μM 5-HT, neuronal cells were exposed to increasing concentrations of ICS 205 930 and MDL 72222. Results are expressed as a percentage of residual stimulation relative to the stimulatory action of 1 μM 5-HT (100%). Data are the mean ± standard error from three separate experiments performed in duplicate.

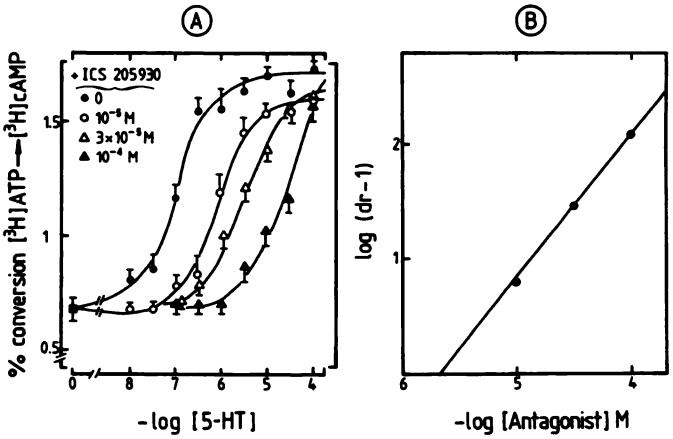

Fig. 4. Antagonism of 5-HT-induced stimulation of cAMP formation by ICS 205 930 in colliculi neurons in primary culture. A, 5-HT concentration-effect curves are represented alone and in the presence of 10, 30, and 100 μM ICS 205 930. Each *point* represents the percent of conversion of [^{3}H]ATP to [^{3}H]cAMP ± standard error (*vertical bars*) performed on three separate experiments each in duplicate. B, Schild plot analysis of the experiment presented in A.

tems. Although the classification of 5-HT receptors has so far only been established from a pharmacological basis, one can expect that the number of different 5-HT receptors is likely to be one of the highest. Indeed, at least four subtypes of 5-HT_1 receptors (5-HT_{1B}, 5-HT_{1b}, 5-HT_{1C}, and 5-HT_{1D}) have been described (1–10, 33) and one type of 5-HT_2 receptor does exist (12). Even the newly introduced 5-HT_3 receptor category already contains three subtypes (19, 20). Furthermore, a great number of 5-HT responses both in the central nervous system (42, 53, 54) and on the periphery (54–58) cannot be recognized as being mediated by classical 5-HT_1, 5-HT_2, or 5-HT_3 receptors. Here we report that, in primary culture of mouse embryo colliculi neurons, 5-HT receptors positively coupled with an adenylate cyclase cannot be classified as being either 5-HT_1 or 5-HT_2 receptors and could not be related to the 5-HT_3 receptor family, despite the antagonist effect of ICS 205 930 (a selective 5-HT_3 antagonist).

Several results indicate that 5-HT receptors positively coupled with an adenylate cyclase are neither of the 5-HT_1 nor 5-HT_2 families. 1) Neither the 5-HT_{1A}-specific agonists (8-OH-DPAT, ipsapirone, or buspirone) (Table I), nor the 5-HT_{1B} agonist RU 24969 had any activity (Fig. 1). 2) 5-CT, another potent specific 5-HT_1 agonist, was 30-fold less potent (EC_{50} = 3160 ± 630 nM) than 5-HT (EC_{50} = 109 ± 17 nM), whereas 5-CT is known to have a similar or an even higher affinity than 5-HT (nanomolar range) on 5-HT_{1A} (26–31), 5-HT_{1B} (32), or 5-HT_{1D} (10, 34) receptors. The affinity of 5-CT for 5-HT_{1C} receptors is lower (micromolar range) (59). However, both the pharmacology of 5-HT_{1C} receptor and its mechanism of action (coupling with a phospholipase C) (36) indicate that it is closer to the 5-HT_2 than to the 5-HT_1 family. 3) *d*-LSD, which has a nanomolar affinity for both 5-HT_1 and 5-HT_2 receptors (1), was a very poor partial agonist (Table 1). 4) Methiothepin, a potent 5-HT_1 and 5-HT_2 antagonist, was completely inactive in inhibiting 5-HT-induced cyclic AMP

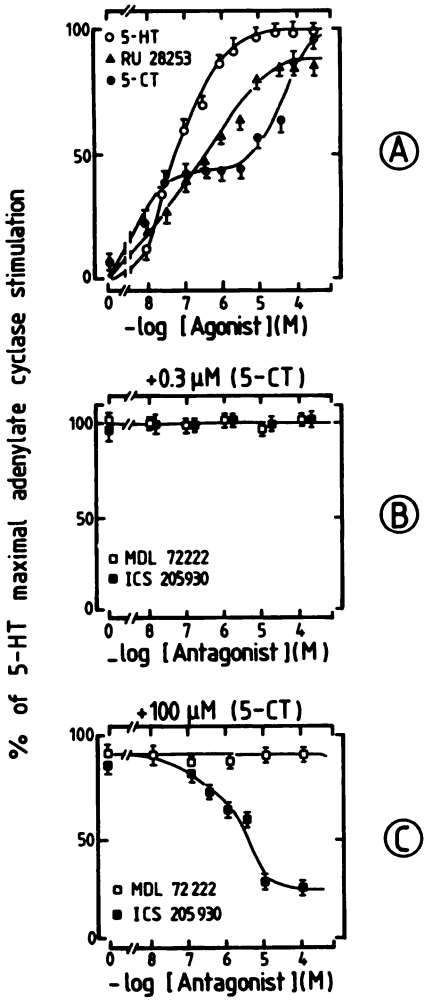

Fig. 5. Stimulation of adenylate cyclase activity and antagonism of 5-CT-mediated stimulatory effect on adenylate cyclase activity by two selective 5-HT_3 antagonists in adult guinea pig hippocampal membranes. A, Concentration-effect curves of 5-HT, 5-CT, and RU 28253. Each *point* represents the cAMP production ± standard error, in triplicate determinations. B, Reversal effect of increasing concentrations of ICS 205 930 and MDL 72222 on the cAMP stimulation induced by 0.3 μM 5-CT. Results are expressed as the percentage of adenylate cyclase activity stimulated by 0.3 μM of 5-CT (100%) at increasing concentrations of antagonists. C, Reversal effect of increasing concentrations of ICS 205 930 and MDL 72222 on the cAMP stimulation induced by 100 μM 5-CT. Results are expressed as the percentage of adenylate cyclase activity relative to the maximum stimulation obtained at 100 μM 5-CT (100%). Basal, 5-CT (0.3 μM) and 5-CT (100 μM) adenylate cyclase activities were 255 ± 11, 468 ± 20, and 720 ± 25 pmol/5 min/mg, respectively in A, whereas in B and C, they were 270 ± 9, 430 ± 22, and 656 ± 15 pmol/5 min/mg, respectively.

production. 5) Ketanserin, a highly selective 5-HT_2 antagonist, was also completely inactive, just like mesulergine, a 5-HT_{1C} antagonist, and (−)-pindolol, a 5-HT_{1B} antagonist (Table 2). 6) Spiperone, a 5-HT_{1A} and 5-HT_2 antagonist, and metergoline, a 5-HT_1 and 5-HT_2 antagonist, were also inactive (Table 2). These pharmacological characteristics present similarities to those described by Shenker *et al.* (31) for one of the 5-HT receptors positively coupled to an adenylate cyclase in guinea pig hippocampus, which has a low affinity for 5-HT and 5-CT and on which none of the classical 5-HT_1 or 5-HT_2 receptor antagonists act (31) (Table 2). The similarity between 5-HT receptors of hippocampus and colliculi neurons is also indicated by the fact that the rank order of potencies for a series of agonists is almost identical in both systems. In hippocampus, Shenker *et al.* (31) found the following order of potency for agonists: 5-HT = 5-MeOT > bufotenine > 5-CT > tryptamine ≫ 8-OH-DPAT; whereas in colliculi neurons, we found the following order of potency: 5-HT = 5-MeOT > RU 28253 > bufotenine > 5-CT > 5-MeO-*N*,*N*-DMT > tryptamine ≫ 8-OH-DPAT. Furthermore, both receptors are insensitive to RU 24969 and sensitive to RU 28253 (Figs. 1 and 5A, Table I).

The possibility that these receptors were of the 5-HT_3 type was then studied. One of the most pertinent results was that ICS 205 930, the specific 5-HT_3 antagonist, was able to inhibit this 5-HT-mediated adenylate cyclase stimulation. In mouse embryo colliculi neurons, ICS 205 930 inhibited the 5-HT-stimulated adenylate cyclase with a K_i equal to 997 ± 353 nM (six experiments), whereas, in guinea pig hippocampal membranes, it was equal to 454 ± 120 nM (three experiments).

At least three 5-HT_3 receptors have been classified thus far (19, 20). 5-HT_{3A} receptors are present on post-ganglionic sympathetic neurons of the superior cervical ganglion and sensory nerves; 5-HT_{3B} receptors are present on sympathetic and parasympathetic nerves; and 5-HT_{3C} receptors are present on enteric nerves of guinea pig ileum. ICS 205 930 has a K_D of 0.39, 0.15, and 10 nM on 5-HT_{3A}, 5-HT_{3B}, and 5-HT_{3C}, respectively (19). Therefore, it is clear that the affinity of ICS 205 930 for the 5-HT receptor positively coupled with an adenylate cyclase described here is 50 to 100 times lower than for 5-HT_{3C} receptors. MDL 72222, another specific 5-HT_{3A} and 5-HT_{3B} receptor antagonist that is inactive on 5-HT_{3C}, is also inactive on 5-HT receptors described in hippocampal membranes (Fig. 5, B and C; Table 2) and almost inactive in colliculi neurons (Fig. 3).

Therefore, the selective 5-HT_3 antagonist ICS 205 930 was the only drug able to inhibit the 5-HT receptors positively coupled with an adenylate cyclase described in this report. The pharmacological profiles of the 5-HT receptors present in colliculi neurons and in hippocampal membranes do not resemble those recently found for the 5-HT_3 binding sites in brain (22) and in neuroblastoma cells (23).

This conclusion can also be reached when one considers the effect of 5-HT_3 agonists on the 5-HT receptor involved in cAMP production in colliculi neurons. The effects of phenylbiguanide (17) and 2-CH_3-5-HT (13), reported in Table 1, indicate that we are not dealing with one of the three 5-HT_3 receptors described thus far. Indeed, phenylbiguanide was completely inactive (Table 1), whereas 2-CH_3-5-HT was at least 10 to 100 times less potent in stimulating cAMP production than in triggering the 5-HT_3 response or in interacting with 5-HT_3 binding sites (13, 19, 20).

It can also be noted that 5-HT receptors positively coupled with an adenylate cyclase, which we found in mouse embryo colliculi neurons and adult guinea pig membranes, are probably very similar to the 5-HT receptor that we described 10 years ago in infant rat brain and adult guinea pig brain (52, 53). Although this last receptor was claimed to be identical to the 5-HT_1 receptors labeled with [^{3}H]5-HT, we have always been against this proposal (53). The fact that there are 5-HT receptors positively coupled with an adenylate cyclase and inhibited specifically by ICS 205 930 is also suggested by a recent study, published as an abstract (60), showing that a 5-HT-stimulated

adenylate cyclase of spinal cord can be inhibited by low concentrations of ICS 205 930.

In conclusion, the 5-HT receptors positively coupled with an adenylate cyclase described in this report, both in primary cultures of mouse embryo colliculi neurons and in adult guinea pig membranes, are certainly not identical to one of the 5-HT_3 receptors described thus far, even if it is only inhibited by a 5-HT_3 specific antagonist. Until further classification of the 5-HT receptors is available, we propose to associate these receptors with a novel category of receptors (5-HT_4).

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