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Pharmacological Characterization of 5-Hydroxytryptamine$_4$(5-HT$_4$) Receptors Positively Coupled to Adenylate Cyclase in Adult Guinea Pig Hippocampal Membranes: Effect of Substituted Benzamide Derivatives

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SUMMARY

Adult guinea pig hippocampal membranes contain two 5-hydroxytryptamine (5-HT) receptors positively coupled with an adenylate cyclase. One is a typical 5-HT$_{1A}$ receptor and the second is a nonclassical 5-HT receptor that we previously proposed to call 5-HT$_4$. Here, we show that 4-amino-5-chloro-2-methoxy-benzamide derivatives are agonists of 5-HT$_4$ receptors in guinea pig hippocampal membranes. Their effects on the adenylate cyclase of these membranes are not additive with those of 5-HT but are additive with those of RU 24969, a typical 5-HT$_1$ agonist. The effects of benzamides, as well as those of 5-HT, on 5-HT$_4$ receptors are not blocked by 5-HT$_1$, 5-HT$_2$, or 5-HT$_3$ antagonists except ICS 205 903, which does so with a low affinity (1 μM). The potency of benzamides (cisapride > BRL 24924 > zacopride > BRL 20627 > metoclopramide) is similar to their effect on 5-HT$_4$ receptors positively coupled with an adenylate cyclase of fetal mouse colliculi neurons.

We have recently described nonclassical 5-HT receptors that we designated 5-HT$_4$ (1–3). These receptors mediate the stimulation of adenylate cyclase activity both in embryo colliculi neurons in primary culture and in adult guinea pig hippocampal membranes (1).

They have a very specific pharmacology. They are neither of the 5-HT$_1$ type, inasmuch as very potent 5-HT$_1$ agonists (8-hydroxy-(2-di-*n*-propylamino)tetralin, RU 24924, ipsapirone) or antagonists (methiothepin, metergoline, spiperone, pindolol, mesulergine) are inactive, nor of the 5-HT$_2$ type, inasmuch as ketanserine has no effect. They are not of the 5-HT$_3$ type, inasmuch as 1) 2-methyl-5-HT was ineffective, whereas analogues of 5-HT substituted in position 5 of the indole ring were full or partial agonists (4), and 2) most of the 5-HT$_3$ antagonists were inactive (BRL 43694, GR 38032F, MDL 72222, cocaine) except ICS 205 930, which has a low potency (about 1 μM). In addition to these properties, we found that, in fetal colliculi neurons, 5-HT$_4$ receptors have the unique ability, among other 5-HT receptors, to be stimulated by 4-amino-5-chloro-2-methoxy-benzamide derivatives including some 5-HT$_3$ antagonists (BRL 24924, zacopride, cisapride, metoclopramide) (3). The pharmacological profile of 5-HT$_4$ receptors in colliculi neurons is very similar to that of one of the two 5-HT receptors involved in the indirect stimulation of smooth muscle contraction in guinea pig ileum (3). In order to be sure that the 5-HT$_4$ receptors of adult animals are similar to the 5-HT$_4$ receptors of fetal colliculi neurons, we have studied in the present work the effect of 4-amino-5-chloro-2-methoxy-benzamide derivatives on the 5-HT$_4$ receptors of adult guinea pig hippocampal membranes.

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Materials and Methods

Adenylate cyclase assay. Male adult guinea pigs received chronic drug treatment with parachloro-phenylalamine (300 mg/kg/day). This treatment was necessary to decrease the contamination of membranes with 5-HT, which occurs if endogenous 5-HT is not lowered before the animals are killed. The animals were treated for 3 days, 2 days, and 1 day before decapitation. The last dose was given 4 hr before decapitation. Hippocampi were homogenized in 10 ml of a solution containing 300 mM sucrose, 20 mM Tris·HCl (pH 7.4), 1 mM EGTA, 5 mM EDTA, and 5 mM dithiothreitol. The homogenate was diluted in 50 ml of the same medium and centrifuged at 40,000 × *g* for 10 min at 0°. The pellet from this centrifugation was resuspended in 4 ml of the same medium. To start the assay, aliquots from the resuspended medium (40 μl) were added to 100 μl of the assay medium. The final composition of the medium was 80 mM Tris·Hcl (pH 7.4), 0.1 mM ATP, 2 mM $MgCl_2$, 10 μM GTP, 1 mM cyclic AMP, 2 mM theophylline, 5 mM creatine phosphate, 0.2 mg/ml creatine kinase, 1–2 μCi of [α-^{32}P]ATP, and 0.01 μCi of [^{3}H]cyclic AMP. The 5-min incubation period at 30° was stopped

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

by adding 900 μl of 5.5 mM Tris·HCl (pH 7.6), 0.4 mM ATP, 0.6 mM cyclic AMP, 10 mM $CaCl_2$, 0.1 N HCl. [^{32}P]cyclic AMP formed and [^{3}H]cyclic AMP were isolated according to the method of Salomon *et al.* (5).

Data analysis. Each figure shows a representative experiment, which was performed at least three times. Experimental points are the means ± standard errors of triplicate determinations. EC_{50} refers to the agonist concentrations yielding 50% of maximal activation determined directly on each concentration-response curve. K_i values were calculated from the concentration of the drug reversing the stimulation obtained with agonists by 50%, using the Cheng-Prusoff equation (6). Computer analysis of the concentration-response curves were performed as previously described (1).

Drugs. The following drugs were generously donated: BRL 24924 [(±)-endo-4-amino-5-chloro-2-methoxy-N-(1-azabicyclo[3.3.1]non-4-yl)benzamide monohydrochloride]; BRL 20627 [(2-α,6β,9aα)-(±)-4-amino-5-chloro-2-methoxy-*N*-(octahydro-6-methyl-2-H-quinolizin)-benzamide HCl], and BRL 43694 [endo-*N*-(9-methyl-9-azabicyclo[3,3,1]non-3-yl)-1-methyl-1*H*-indazol-3-carboxamide] (G. J. Sanger,Beecham Pharmaceuticals, Harlow, UK); cisapride [(*cis*)-4-amino-5-chloro-*N*- [1-[3-(4-fluorophenoxy)propyl]-3-methoxy-4-piperidinyl]-2-methoxy-benzamide], ketanserin and spiperone (Janssen Pharmaceutica, Belgium); metoclopramide (Laboratoires Delagrange, Paris, France); zacopride [4-amino-*N*-(1-azabicylo[2.2.2]oct-3yl)-5-chloro-2-methoxy-benzamide HCl] (Laboratoires Delalande, Rueil-Malmaison, France); RU 24924 (Roussel-Uclaf, Romainville, France); and mesulergine, and SDZ 21099 (Sandoz Ltd. Basel, Switzerland). The following drugs were purchased: 5-HT (Sigma Chemical Co., St. Louis, MO) and ICS 205 930 [(3α-tropanyl)-1*H*-indole-3-carboxylic acid ester] (Research Biochemicals Incorporated Natick, MA).

Results

Effect of 5-HT and 4-amino-5-chloro-2-methoxy-substituted benzamide derivatives on adenylate cyclase activity of guinea pig hippocampal membranes. 5-HT stimulated adenylate cyclase of the guinea pig hippocampal membranes in a concentration-dependent manner (Fig. 1). As previously reported (1, 7), the 5-HT concentration-response curve was shallow. We analyzed this curve, as described in Materials and Methods, and found that it can be fitted according to a model describing the action of 5-HT on two independent receptors. The E_{max} values of the high affinity receptor (R_H) and the low affinity receptor (R_L) were 42 and 58% of the maximal 5-HT stimulation, respectively, whereas the EC_{50} values were 36 and 295 nM, respectively. These values agree with those previously obtained in this system (1). Very similar results were obtained by Shenker *et al.* (7). R_H receptors are of the 5-HT$_{1A}$ type (1, 7), whereas R_L receptors are those we designated as 5-HT$_4$ receptors. Fig. 1 also shows that the 4-amino-5-chloro-2-methoxy-substituted benzamides tested were able to stimulate adenylate cyclase of guinea pig hippocampal membranes. Cisapride was more potent (EC_{50}: 210 ± 34 nM; five determinations) than BRL 24924 (EC_{50}: 33 ± 0.22 μM; five determinations). Zacopride had a lower potency than BRL 24924, which was difficult to determine because concentrations higher than 10^{-4} M could not be reached because of the solubility problem. Metoclopramide was a very weak agonist (2–3% of the E_{max} reached with 5-HT). Nonbenzamide 5-HT$_3$ antagonists such as BRL 43694 (8) and GR 38032 F were inactive (data not shown).

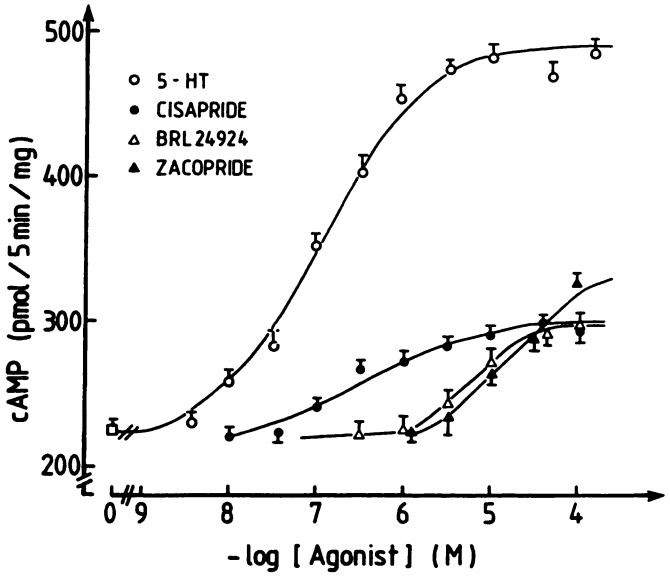

Fig. 1. Stimulation of adenylate cyclase activity of guinea pig hippocampal membranes by 5-HT and several benzamide derivatives.

Effects of 5-HT and benzamide derivatives on adenylate cyclase activity of guinea pig hippocampal membranes are not additive. Saturating concentrations of benzamides (cisapride, BRL 24924, and zacopride) were tested alone and in the presence of a saturating concentration of 5-HT. Clearly, the benzamide and 5-HT effects were not additive, suggesting that benzamides act on one of the 5-HT receptors coupled with adenylate cyclase in this system (Fig. 2). In other experiments (three determinations), we have measured the amounts of cAMP produced in the presence of 5-HT (50 μM), isoproterenol (10 μM), and both agonists. They were equal to 143 ± 9, 62 ± 6, and 225 ± 15, pmol/5min./mg, respectively, indicating that in this system additivity between the effects of independent receptors coupled to adenylate cyclase can be obtained.

Additive effects of RU 24969 and benzamide derivatives on adenylate cyclase activity of guinea pig hippocampal membranes. We have previously reported that RU

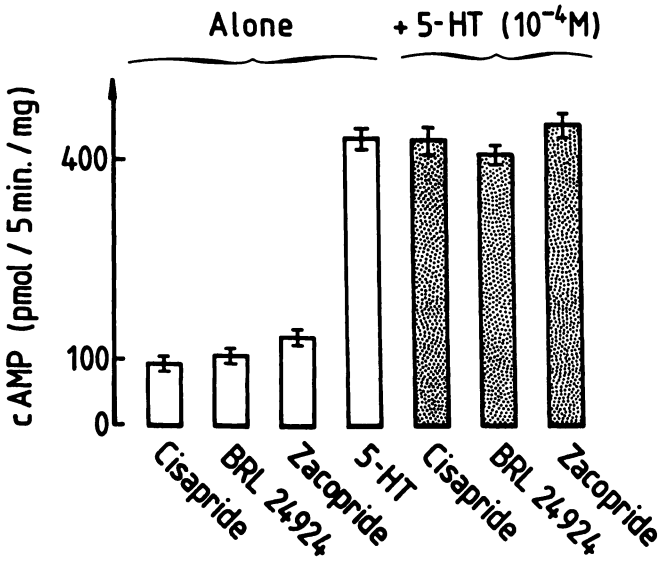

Fig. 2. Nonadditive effects of some benzamide derivatives and 5-HT on adenylate cyclase activity of guinea pig hippocampal membranes. The concentrations of cisapride, BRL24924, and zacopride were 10, 10, and 100 μM, respectively. Basal adenylate cyclase activity was 210 ± 16 pmol/5 min/mg.

24969 specifically stimulated the 5-HT_{1A} receptors coupled with adenylate cyclase in guinea pig hippocampal membranes and that its action was blocked by low doses of spiperone (1) (see also Fig. 4). In order to know whether benzamides act via 5-HT_{1A} or 5-HT_4 receptors, we tested the possible additive effects of maximal doses of RU 24969 and benzamides (cisapride or zacopride). As seen in Fig. 3, the effects of RU 24969 and benzamides were additive, suggesting an action of benzamides on 5-HT_4 receptors and not on 5-HT_{1A} components.

Effects of spiperone and ICS 205 930 on 5-HT-, RU 24969-, and benzamide-stimulated adenylate cyclase activity. As expected, the 5-HT stimulation of adenylate cyclase was partially blocked by both ICS 205 930 and spiperone (Fig. 4A). A combination of both antagonists completely blocked the 5-HT response (Fig. 4A). Although the 5-HT_4 component is generally slightly higher than 50% (1) (Fig. 1), the maximal inhibition obtained in this experiment with ICS 205 930 was slightly lower than that obtained with spiperone. This could be explained by the fact that 5-HT was used at a concentration of 1 μM (a submaximal dose). At this concentration, the percentage of occupancy of 5-HT_{1A} receptors (EC_{50}, 23 nM) (1) was 98%, whereas it was 80% for 5-HT_4 receptors (EC_{50}, 263 nM) (1). The K_i values for spiperone and ICS 205 930 calculated considering the relative EC_{50} of 5-HT for 5-HT_{1A} and 5-HT_4 receptors were equal to 16 and 540 nM, respectively. These values are very close to those we previously found (1).

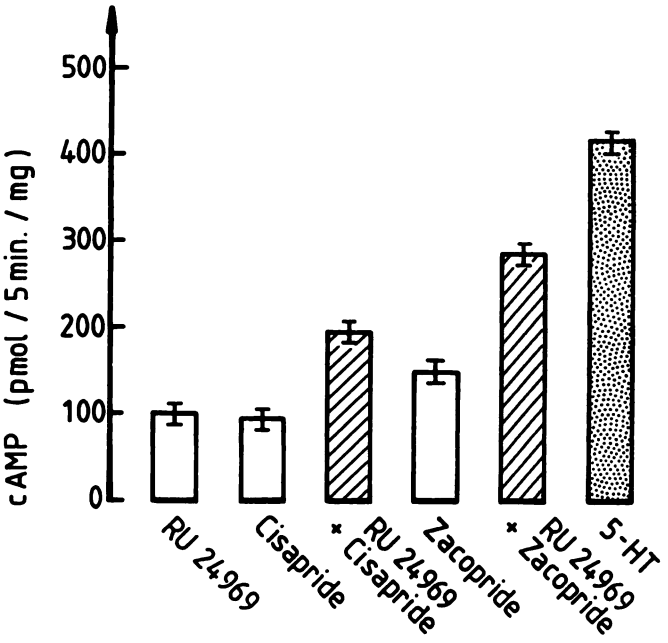

Fig. 3. Additive effects of RU 24969 and some benzamide derivatives on adenylate cyclase activity of guinea pig hippocampal membranes. The concentrations of RU 24969, cisapride, zacopride, and 5-HT were 1, 10, 100, and 10 μM, respectively.

As expected, RU 24969 stimulation was blocked by spiperone, whereas benzamide activations were not affected by this specific 5-HT_{1A} antagonist (Fig. 4B). In contrast, ICS 205 930 completely blocked benzamide stimulations without affecting RU 24969 stimulation (Fig. 4C). The K_i of ICS 205 930 in blocking the cisapride response was 500 nM (Fig. 4C).

Effects of a high dose (10 μM) of ketanserin, mesulergine, and SDZ 21009 on RU 24969- and benzamide-stimulated adenylate cyclase. Whereas a high dose (10 μM) of 5-HT_2, 5-HT_{1C}, and 5-HT_{1B} antagonists was able to suppress the 5-HT_{1A} response to submaximal doses of RU 24969 (10^{-7} M) (which is expected, according to the relative affinities of these compounds for 5-HT_{1A} receptors), they were unable to affect the response to submaximal doses of benzamides (Fig. 5).

Discussion

It is now clear from the report by Shenker *et al.* (7), our previous work (1), and the present data that 5-HT stimulates two distinct 5-HT receptors positively coupled with an adenylate cyclase in adult guinea pig hippocampus. The first one has a high affinity for 5-HT (20–50 nM) and possesses the pharmacological characteristics of 5-HT_{1A} receptors (Refs. 1 and 7, and present results). The second one has a lower affinity for 5-HT (200–500 nM) (1, 7) and has been called R_L by Shenker

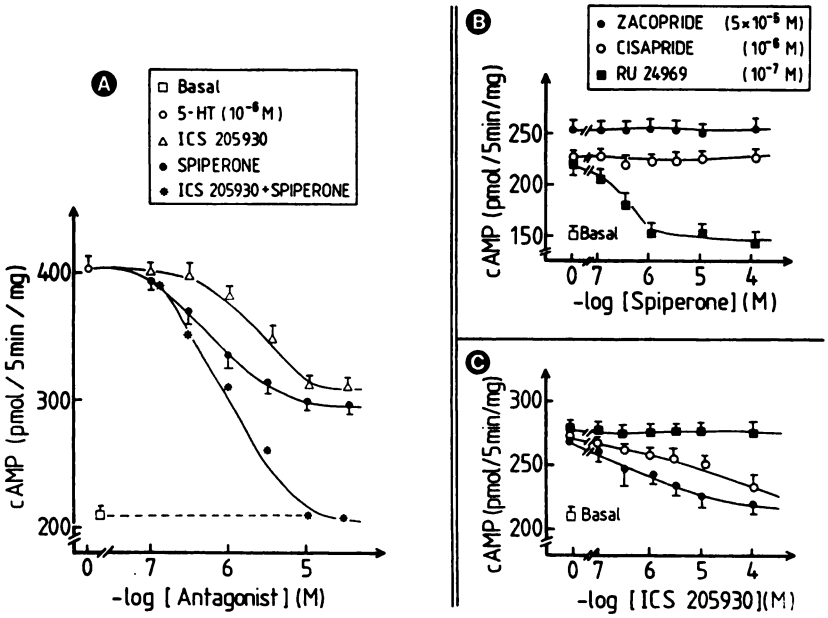

Fig. 4. Effects of spiperone and ICS 205 930 on 5-HT and benzamide stimulation of adenylate cyclase.

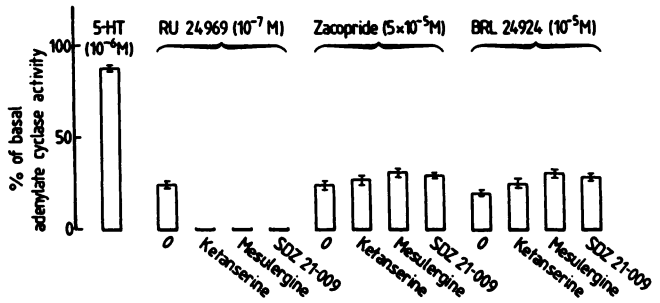

Fig. 5. Effect of a high dose (10 μM) of different 5-HT antagonists on RU 24969-, zacopride-, and BRL 24924-stimulated adenylate cyclase. Basal adenylate cyclase activity was 180 ± 13 pmol/5 min/mg of protein.

et al. (7) and 5-HT$_4$ by Dumuis *et al.* (1). 5-HT$_4$ receptors are not blocked by any classical 5-HT antagonists, except ICS 205 930, which does so with a low affinity (500–1000 nM) (1). A combination of spiperone (a 5-HT$_{1A}$ antagonist) and ICS 205 930 completely blocked the 5-HT response (Fig. 4A). We have shown that these 5-HT$_4$ receptors are present in primary cultures of fetal mouse colliculi neurons (1). In addition, we have shown that the 5-HT$_4$ receptors of this system are stimulated by a series of 4-amino-5-chloro-2-methoxy-benzamide derivatives. Here, we also show that these benzamides are able to stimulate the adenylate cyclase of guinea pig hippocampus. The order of potency of benzamides in this system is cisapride ≥ 5-HT > BRL 24924 > zacopride > metoclopramide, which is very close to the order found in mouse colliculi neurons (3).

The benzamide effect is not additive with that of 5-HT but is additive with that of RU 24969. This indicates that benzamides do not stimulate the 5-HT$_{1A}$ part of the 5-HT response but rather the 5-HT$_4$ part. This was confirmed by the blockade of benzamide stimulation by ICS 205 930 with a potency (500 nM) similar to the one blocking the 5-HT$_4$ response (1) and by the absence of blockade by spiperone (Fig. 4). Note that other 5-HT$_1$, 5-HT$_2$, or 5-HT$_3$ antagonists, even at very high doses, were unable to block benzamide responses (Fig. 5). Some differences appear between the colliculi and hippocampal systems when the efficacies of these drugs are compared. In fetal colliculi neurons, cisapride, BRL 24924, and zacopride are more efficacious than 5-HT itself, whereas metoclopramide is a partial agonist (3). In contrast, in guinea pig hippocampal membranes, all benzamides were partial agonists. Indeed, experiments shown in Figs. 1 and 4 as well as previous results (1) indicate that the 5-HT$_4$ component represents at least 50% of the cyclic AMP produced in the presence of 5-HT. The effects of benzamides were never higher than 20–25% of the cyclic AMP produced in the presence of 5-HT.

Not only are the 5-HT$_4$ receptors of adult hippocampal membranes similar to the 5-HT$_4$ receptors of fetal mouse colliculi neurons but they are also similar to one of the 5-HT receptor types involved in guinea pig ileum contraction and more generally in gastroenteric contractions of several species (9–15).

At least two indirect mechanisms exist for guinea pig ileum contractions triggered by two different 5-HT receptor types (4, 10, 14). Both are present on intramural nerve terminals and stimulate the release of neurotransmitters able to contract smooth muscles. One is the 5-HT$_3$ type, because it is blocked by very low concentrations of ICS 205 930 (nanomolar concentrations) and other classical 5-HT antagonists, stimulated by 2-methyl-5-HT, and resistant to analogues of 5-HT substituted at position 5 (4, 9, 12). Its action on neurotransmitter release is also relatively resistant to morphine and atropine (9, 12). The second 5-HT receptor type of guinea pig ileum that is involved in contraction has a pharmacology that greatly resembles that of the 5-HT$_4$ receptor we describe in the central nervous system.

Like the 5-HT$_4$ type, the receptor of guinea pig ileum is blocked by micromolar concentrations of ICS 205 930 (13), sensitive to 5-HT analogues substituted at position 5 (13), stimulated by 4-amino-5-chloro-2-methyl-benzamide derivatives, and insensitive to cocaine (1, 10, 11, 13, 14). It is localized on cholinergic terminals, inasmuch as 5-HT, cisapride, and metoclopramide increase acetylcholine release (16) and their action is potently blocked by atropine acting postsynaptically and morphine acting presynaptically (9, 11–13).

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