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Pharmacological comparison between [^{3}H]GR 113808 binding sites and functional 5-HT$_4$ receptors in neurons

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Abstract

5-HT$_4$ receptors positively coupled to adenylyl cyclase and possessing unique pharmacological properties were first described in mouse colliculi neurons using functional studies. The recent introduction of a radiolabeled 5-HT$_4$ receptor antagonist, [^{3}H]GR 113808 [1-[2-(methylsulphonylamino)ethyl]4-piperidinyl]methyl-1-methyl-indole-3 carboxylate] having high specificity and affinity allowed the pharmacological comparison between the specific binding sites identified with this compound and the functional 5-HT$_4$ receptors in the same preparation, the colliculi neurons. We show here that [^{3}H]GR 113808 binding is saturable in this preparation and reveals a homogeneous population of sites with a pK_d value of 9.5 ± 0.2 and a B_{max} of 75 ± 23 fmol/mg protein. Seventeen agonists and six antagonists with molecules structurally related either to indoles, benzamides or benzimidazolones and previously known as 5-HT$_4$ receptor ligands, were tested for their ability to compete with [^{3}H]GR 113808 binding sites and to stimulate or inhibit 5-HT-stimulated adenylyl cyclase activity. Highly significant correlations were obtained between the affinities of either agonists or antagonists for [^{3}H]GR 113808 binding sites and their potencies for functional 5-HT$_4$ receptors (r = 0.87 and 0.99, respectively). In addition, we also found good correlations between the K_d of several 5-HT$_4$ receptor ligands determined in cell membranes of mouse colliculi neurons and their K_d determined in previous studies in guinea-pig striatum (0.95) and in human caudate (0.97). [^{3}H]GR 113808 binding studies demonstrated that the 50% decrease in 5-HT-stimulated cAMP accumulation which followed a 5 min exposure period with 5-HT (10 μM) was not accompanied by any significant decrease in the number of binding sites. Longer exposure periods with 5-HT resulted in a decrease in [^{3}H]GR 113808 binding sites which started to be significant after 30 min.

Keywords: 5-HT$_4$ receptor; [^{3}H]GR 113808; Radioligand binding; Desensitization

1. Introduction

The pharmacological profile of 5-HT$_4$ receptors was first established in mouse colliculi neurons and guinea-pig brain using functional studies (Dumuis et al., 1988; Bockaert et al., 1990). Later, this receptor was identified in a wide variety of tissues and species: guinea-pig ileum (Craig and Clarke, 1990), colon (Elswood et al., 1991), rat oesophagus (Baxter et al., 1991), frog and human adrenocortical cells (Idres et al., 1991; Lefèbvre et al., 1992), human urinary bladder (Corsi et al., 1991), human and porcine heart (Kaumann, 1990; Villalòn et al., 1990; Kaumann et al., 1991; Ouadid et al., 1992). Very recently, the expression of 5-HT$_4$ receptors was described in human brain (Monferini et al., 1993; Waeber et al., 1993; Domenech et al., 1994). The pharmacology of the 5-HT$_4$ receptor clearly differs from that of the other 5-HT receptors (for recent reviews see Bockaert et al., 1992; Clarke and Bockaert, 1993; Ford and Clarke, 1993; Sanger and Gaster, 1994). Derivatives of three main chemical families have been found to be agonists of the 5-HT$_4$ receptor: indoles, 2-methoxy-4-amino-5-chloro benzamides and benzimidazolones. However, all these agonists have some activity on other receptors. Indoles act as agonists on various 5-HT receptor subtypes, whereas most of the benzamides and benzimidazolones act as 5-HT$_3$ receptor antagonists. Molecules structurally related to benzamides or benzoates (SB 205149, SC-53116, SC-49518 and ML 10302) have been described as the most potent and selective 5-HT$_4$ receptor agonists (Flynn et al., 1992; Baxter et al., 1993; Gullikson et al., 1993; Langlois et al., 1994). Since the discovery of tropisetron as a 5-HT$_3$/5-HT$_4$ receptor antagonist, other 5-HT$_4$ receptor antagonists have been found, most of them belonging to one of the three

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chemical families defined above (Sanger and Gaster, 1994). Two compounds having a very high selectivity for 5-HT_4 receptors were used to develop radioligands: [^{3}H]GR 113808 (Grossman et al., 1993) and the seven iodo analogues of SB 204070, named [^{125}I]SB 207710 (Brown et al., 1993; Grossman et al., 1993; Gaster and Sanger, 1994; Wardle et al., 1994). Both these compounds have been useful to study brain distribution of the 5-HT_4 receptor binding sites (Grossman et al., 1993; Waeber et al., 1993, 1994; Wardle et al., 1993; Domenech et al., 1994; Jakeman et al., 1994).

The aim of the present study was to compare the pharmacological profile of [^{3}H]GR 113808 binding sites in membranes of mouse colliculi neurons on the one hand, with the pharmacological profile of the functional 5-HT_4 receptors positively coupled to adenylyl cyclase on the other hand. Such a comparison has never been done on the same preparation and 5-HT_4 receptors in colliculi neurons are certainly those for which the pharmacological properties are the most completely known.

2. Materials and methods

2.1. Neuronal cultures

Cells generated from colliculi of 14–15-day-old Swiss mouse embryos were dissociated and plated in serum-free medium in Falcon petri dishes (1.5×10^6 cells/ml) previously coated successively with poly-L-ornithine and culture medium containing 10% fetal calf serum. Cultures were maintained for 9–11 days at 37°C in a humidified atmosphere in 6% CO_2/94% air. Under these culture conditions previously described in detail (Weiss et al., 1986), 95% of the cells are neurons.

2.2. Cyclic AMP formation

After 9 days in vitro, intracellular cAMP levels were determined in colliculi neurons by measuring the conversion of the [^{3}H]adenine nucleotide precursor, [^{3}H]ATP to [^{3}H]cAMP, as previously described (Dumuis et al., 1988).

2.3. Desensitization of the 5-HT_4 receptor-adenylyl cyclase system in colliculi neurons

Regardless of the duration of the desensitization period (0–24 h), neurons were incubated in culture medium containing 2 μCi/ml [^{3}H]adenine for 2 h prior to the cAMP accumulation period which immediately followed the desensitization period. The cAMP accumulation was started by adding culture medium containing 3-isobutyl-1-methyl-xanthine (IBMX) (0.75 mM) plus forskolin (0.1 μM) and cAMP-stimulating agents. The accumulation was measured for 5 min as described above (cAMP accumulation period). During the desensitization period, performed in the absence of IBMX and forskolin, no significant increase was observed in the presence of 5-HT.

2.4. Membrane preparations

After 9 days in cultures, cells were scraped and harvested in phosphate-buffered saline and then centrifuged at 4°C, $900 \times g$ for 4 min. The cell pellet was resuspended in a buffer containing 10 mM Hepes (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]) (pH 7.4), 5 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid), 1 mM EDTA (ethylenediaminetetraacetic acid) and 0.32 M sucrose and homogenized with a glass-Teflon homogenizer at 4°C (10 shakes). The homogenate was centrifuged at $20\,000 \times g$ for 20 min, the membrane pellet was resuspended in 50 mM Hepes (pH 7.4) (5 mg of protein in 1 ml solution) and stored at −25°C until used as described previously (Waeber et al., 1993).

2.5. Radioligand binding studies

Membrane suspension (100 μl), prepared as described above, was diluted 5 times with 50 mM Hepes (pH 7.4) (50–100 μg of protein) containing 10 μM pargyline and 0.01% ascorbic acid and was incubated at 37°C for 30 min with 100 μl [^{3}H]GR 113808 (specific activity: 83 Ci/mmol) and 50 μl of buffer or competing drugs. For saturation analysis assays, various concentrations of [^{3}H]GR 113808 (0.001–1 nM) were used. The incubation was terminated by rapid filtration on Whatman GF/B filters presoaked with 0.1% polyethyleneimine; the filters were washed with 3×4 ml of ice-cold buffer (10 mM Hepes). Total duration of the filtration was less than 10 s. Radioactivity was counted using a complete phase combining system for liquid scintillation counting (Amersham, Les Ullis, France). Protein concentration in the samples was determined using the Bio-Rad protein assay (Bradford, 1976).

2.6. Data analysis

Competition and saturation experiments were analyzed by non-linear regression using the computer program LIGAND (Munson and Rodbard, 1980). Saturation experiments were also analyzed according to Scatchard (Scatchard, 1949). IC_{50} values required to displace 50% of [^{3}H]GR 113808 binding were converted to K_d values according to the equation (Cheng and Prussoff, 1973):

$$K_d = IC_{50}/1 + S/K_{dS}$$

where S is the [^{3}H]GR 113808 concentration and K_{dS} the equilibrium constant of [^{3}H]GR 113808.

Statistical differences were analyzed by using the Student's *t*-test. Values were considered significantly different when $P < 0.05$.

2.7. Drugs

GR 113808 [1-[2(methylsulphonyl-amino)ethyl]4-piperidinyl]methyl-1-methyl-indole-3 carboxylate, maleate) was synthesized and generously donated by Glaxo (UK); [^{3}H]GR 113808 was synthesized by Amersham (UK) and donated by Glaxo; 5-HT (serotonin) and 5-MeOT (5-methoxytryptamine) were purchased from Sigma Chemical Corp., St. Louis, MO, USA. The following drugs were generously donated: cisapride (*cis*-4-amino-5-chloro-*N*-[1-[3-(4-fluoro-phenoxy)propyl]-3-methoxy-4-piperidinyl]-2-methoxy benzamide) and RN 76186 (*cis*-4-amino-5-chloro-*N*-[1-[4-(4-dimethyl-amino)-1-piperidinyl]-4-oxo-butyl]-3-methoxy-4-piperidinyl]-2-methoxybenzamide) (Janssen Pharmaceutica, Beerse, Belgium); RU 28253 (Roussel Uclaf, Romainville, France); (*R* + *S* −)zacopride ((*R* + *S* −)-4-amino-*N*-(1-azabicyclo[2.2.2]oct-3yl)-5-chloro-methoxybenzamide HCl, (*S*)-Zacopride((*S* −)-4-amino-*N*-(1-aza-bicyclo[2.2.2]oct-3-yl)-5-chloro-2-meth-oxybenzamide HCl), (*R*)-zacopride ((*R* +)-4-amino-*N*-(1-azabicyclo[2.2.2]oct-3-yl)-5-chloro-2-methoxy-benzamide HCl) (Delalande, Paris, France); BIMU 1 (*endo*-*N*-8-

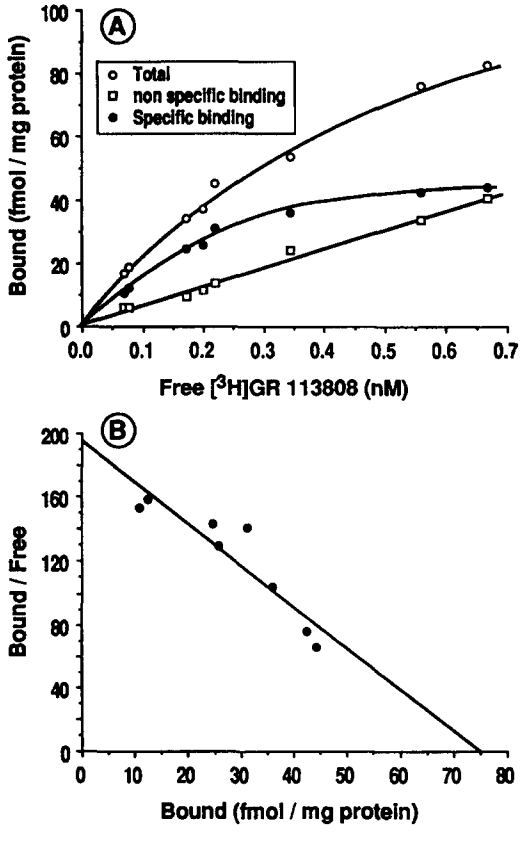

Fig. 1. Characteristics of [^{3}H]GR 113808 binding in membranes of colliculi neurons. (A) Saturation analysis of specific [^{3}H]GR 113808 binding to membranes prepared from colliculi neurons in culture. Data points show total, specific and non-specific binding obtained at different [^{3}H]GR 113808 concentrations (0–0.67nM) in neuronal membranes. Non-specific binding was determined by the addition of 50 μM 5-HT. (B) Scatchard analysis of saturation binding data.

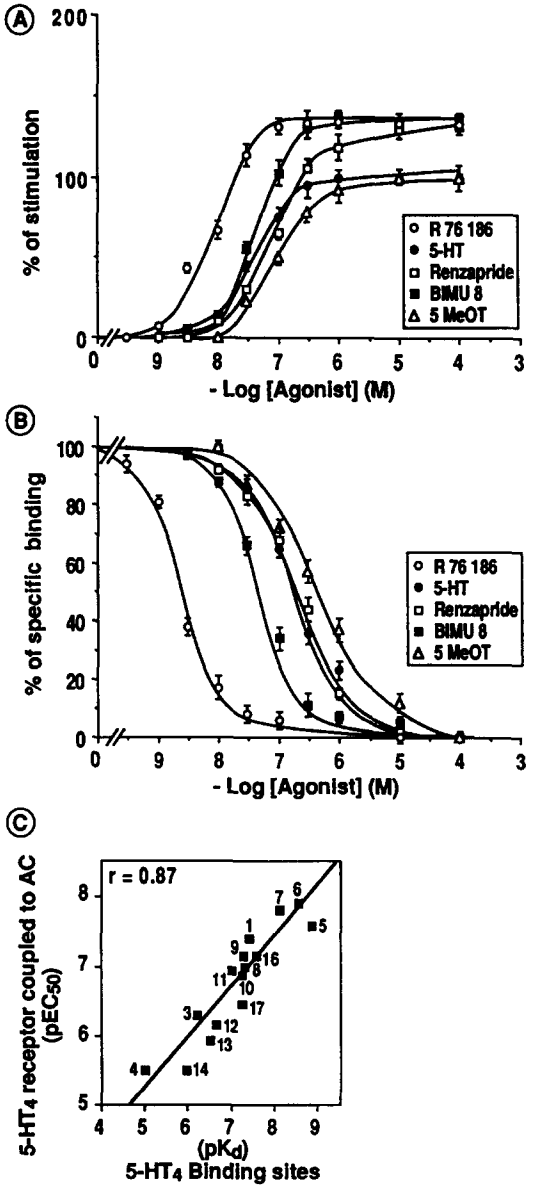

Fig. 2. Relationship between the pharmacological profiles of 5-HT$_4$ receptor agonists that stimulate cAMP production in colliculi neurons and of [^{3}H]GR 113808 binding sites in membranes of colliculi neurons. Panel A represents stimulation of cAMP formation in colliculi neurons by five 5-HT$_4$ receptor agonists: R 76186 (a cisapride analogue) 5-HT, renzapride, BIMU 8 and 5-MeOT. Cells were incubated with increasing concentrations of each agonist and the conversion of [^{3}H]ATP to [^{3}H]cAMP was determined after 5 min at 37°C. In the absence of agonist the percentage of conversion was 1.2±0.15 (n = 16). Results are expressed as the percentage stimulation over basal conversion (means± S.E.M.) performed on four separate experiments each in duplicate. Panel B represents competition experiments carried out in cell membranes of colliculi neurons and using 0.2 nM [^{3}H]GR 113808. The agonists tested were similar as in panel A. Results are expressed as a percentage of the specific binding in the absence of a competing agent and are the means ± S.E.M. of four separate experiments. Panel C represents correlation between the affinities of a series of agonists for [^{3}H]GR 113808 binding sites and the affinities of the same drugs for stimulating cAMP production in colliculi neuronal cells. The pK_d values of 15 compounds (X axis) for [^{3}H]GR 113808 binding sites are correlated with the pEC$_{50}$ values for agonists (Y axis) that stimulate 5-HT$_4$ receptor-mediated cAMP production. All the values are taken from Table 1.

methyl-8-azabicyclo(3.2.1.)oct-3-yl)-2,3-dihydro-3-ethyl-2-oxo-1*H*-benzimidazol-1-carboxamide hydrochloride, BIMU 8 (*endo*-*N*-8-methyl-8-azabicyclo (3.2.1) oct-3-yl)-2,3-dihydro-3-isopropyl-2-oxo-1*H*-benzimidazol-1-carboxamide-hydrochloride, DAU 6215 (endo-*N*-8-methyl-8-azabicyclo (3.2.1) oct-3-yl)-2,3-dihydro-2-oxo-1*H*-benzimidazol-1-carboxamide-hydrochloride, DAU 6285 (*endo*-6-methoxy-8-methyl-8-azabicyclo (3.2.1) oct-3-yl)-2,3-dihydro-2-oxo-1*H*-benzimidazole-1 carboxylate hydrochloride and GR 127487 ([1-[2-(methylsulphonylamino)ethyl]-4-piperidinyl]methyl-5-fluoro-2-methoxy-1*H*-indole-3-carbxylate, hydro-chloride) (Boehringer Ingelheim, Milan, Italy); SDZ 205 557 (2-methoxy-4-amino-5-chlorobenzoic acid 2-(diethylamino) ethyl ester, hydrochloride) and Tropisetron (ICS 205 930) ([(3a-tropanyl)-1*H*-indole-3-carboxylic acid ester] (Sandoz Pharma, Basel, Switzerland); SC-49515 (4-amino-5-chloro-*N*-[*exo*-(hexahydro-1*H*-pyrrolizin-1-yl)-methyl]-2-methoxybenzamide and SC-53116 (1*S*,8*S*)-4-amino-5-chloro-*N*-[*exo*-(hexahydro-1*H*-pyrrolizin-1-yl)methyl]-2-methoxy benzamide (Searle and C.O., Skokie, IL, USA); renzapride ([(±)-*endo*]-2-methoxy-4-amino-5-chloro-*N*-(1-azabicyclo-[3.3.1]-non-4-yl)benzamide mono-hydrochloride) and SB 205149 ([*exo*-*N*-1-butyl-1-azabicyclo-[3.3.1]-nonan-3-yl]-2-methoxy-4-amino-5-chloro)benzamide mono-hydrochloride) (Beecham, Harlow, UK); ML 10302 (2-(1-piperidinyl)ethyl

Table 1
Pharmacological profile of 5-HT_4 receptors present in colliculi neurons in culture

	5-HT_4 binding sites			5-HT_4 receptor coupled to adenylyl cyclase	
	No.	pK_d	±(S.E.M.)	pEC_{50}	±(S.E.M.)
5-HT_4 receptor agonists					
Indoles					
5-HT	1	7.1	(0.11)	7.47	(0.25)
5-MeOT	2	6.28 *	(0.17)	7	(0.10)
RU 28253	3	6.22	(0.28)	6.3	(0.18)
5-CT	4	5.01	(0.20)	5.5	(0.20)
Benzamides					
RN 76186	5	8.86	(0.05)	7.57	(0.13)
SC 53116	6	8.1	(0.08)	7.8	(0.22)
SB 205149	7	7.43	(0.10)	7.4	(0.12)
SC 49518	8	7.3	(0.12)	7	(0.10)
Cisapride	9	7.29	(0.24)	7.15	(0.28)
Clebopride	10	7.27	(0.18)	6.87	(0.13
Renzapride	11	7.01	(0.12)	6.94	(0.25)
Zacopride (*S*)	12	6.65	(0.15)	6.15	(0.18)
Zacopride (*RS*)	13	6.52	(0.10)	5.93	(0.23)
Zacopride (*R*)	14	5.97	(0.22)	5.5	(0.16)
Metoclopramide	15	5.4 *	(0.18)	5.35	(0.20)
Benzimidazolones					
BIMU 8	16	7.6	(0.18)	7.15	(0.09)
BIMU 1	17	7.27	(0.15)	6.45	(0.11)
	No.	pK_d	±(S.E.M.)	pK_i	±(S.E.M.)
5-HT_4 receptor antagonists					
Indoles					
GR 125487	18	10.42	(0.10)	10.6	(0.15)
GR 113808	19	9.5	(0.20)	9.5	(0.20)
ICS 205930	20	6.8	(0.30)	6	(0.25)
Benzoates					
SDZ 205557	21	8.2	(0.22)	7.2	(0.15)
Benzimidazolones					
DAU 6285	22	7.57	(0.15)	6.8	(0.18)
DAU 6215	23	6.65	(0.25)	5.6	(0.23)

cAMP accumulation was measured as described in Methods and in the legend to Fig. 2. EC_{50} values were determined graphically and corresponded to the concentrations of agonists required to obtain a half-maximal stimulation of adenylyl cyclase. Data are expressed as pEC_{50} means ± S.E.M in duplicate determinations performed at least in four separate cultures. For the antagonists, K_i values were calculated from the concentration of the drugs that reversed 50% of the stimulation induced by 5-HT (1 μM). Data are expressed as pK_i means ± S.E.M. of three to five separate cultures performed in duplicate. Binding data correspond to the affinities of various compounds that compete for [^{3}H]GR 113808 binding in homogenates. IC_{50} values correspond to a displacement of 50% of [^{3}H]GR 113808 binding determined experimentally as described in Methods and converted to K_d values according to the equation of Cheng and Prusoff. Data are expressed as pK_d means ± S.E.M. of at least four separate experiments. The concentration of [^{3}H]GR 113808 was 0.2 nM.
* The Hill coefficient was significantly less than 1 ($P < 0.05$). * For these compounds pIC_{50} are given instead of pK_d because the Hill number of the displacement curve is not equal to 1 and the Cheng and Prusoff equation cannot be used. In addition, they are not introduced in the correlation of Fig. 2C.

4-amino-5-chloro-2-methoxybenzoate) (Langlois, CNRS-BIOCIS, Châtenay-Malabry, France).

3. Results

3.1. Saturation experiments

We have previously shown in guinea-pig brain membranes that the density of [^{3}H]GR 113808 specific binding was identical when determined at 25°C or 37°C, although the ratio-specific/non-specific binding was better at 25°C (Waeber et al., 1993). Here we have chosen to perform binding studies on colliculi neuronal membranes at 37°C because functional studies of 5-HT$_4$ receptors are performed at 37°C on the same preparation. At this temperature, the forward binding rate was very fast. In the presence of 0.2 nM of radioligand, maximal binding was achieved within the first 5 min and the ratio of specific/non-specific binding was equal to 2.6. Specific binding as the function of [^{3}H]GR 113808 concentration was a saturable process (Fig. 1A). The Scatchard plot of the dose-binding curve produced only one straight line and gave a pK_d of 9.5 ± 0.2 (n = 6). This pK_d value was similar to values found in human, guinea-pig and rat brain (Grossman et al., 1993; Waeber et al., 1993) (Fig. 1B). The total binding capacity in colliculi neuronal membranes was 75 ± 23 fmol/mg protein (n = 6) (Fig. 1B).

3.2. Relationships between the pharmacological profiles of 5-HT$_4$ receptors that stimulate cAMP production in colliculi neurons and [^{3}H]GR 113808 binding sites in membranes from the same cells

In order to verify that functional 5-HT$_4$ receptors show the same pharmacological characteristics as [^{3}H]GR 113808 binding sites, we have compared the binding affinities of a series of agonists and antagonists, each structurally related to one of the three main classes: indole, benzamide and benzimidazolone with their potencies to activate basal or inhibit 5-HT-stimulated adenylyl cyclase. In Fig. 2 the affinity profiles of 5-HT$_4$ receptor-mediated stimulation of cAMP and the competition curves are shown, using the most representative 5-HT$_4$ agonists. The rank orders of potency for these agonists in stimulating cAMP formation and in binding experiments were R 76186 (a cisapride analogue) > 5-HT > BIMU 8 > renzapride > 5-MeOT (Fig. 2A) and R 76186 > BIMU 8 > 5-HT > renzapride > 5-MeOT (Fig. 2B), respectively. The affinities of the tested agonists including the natural transmitter,

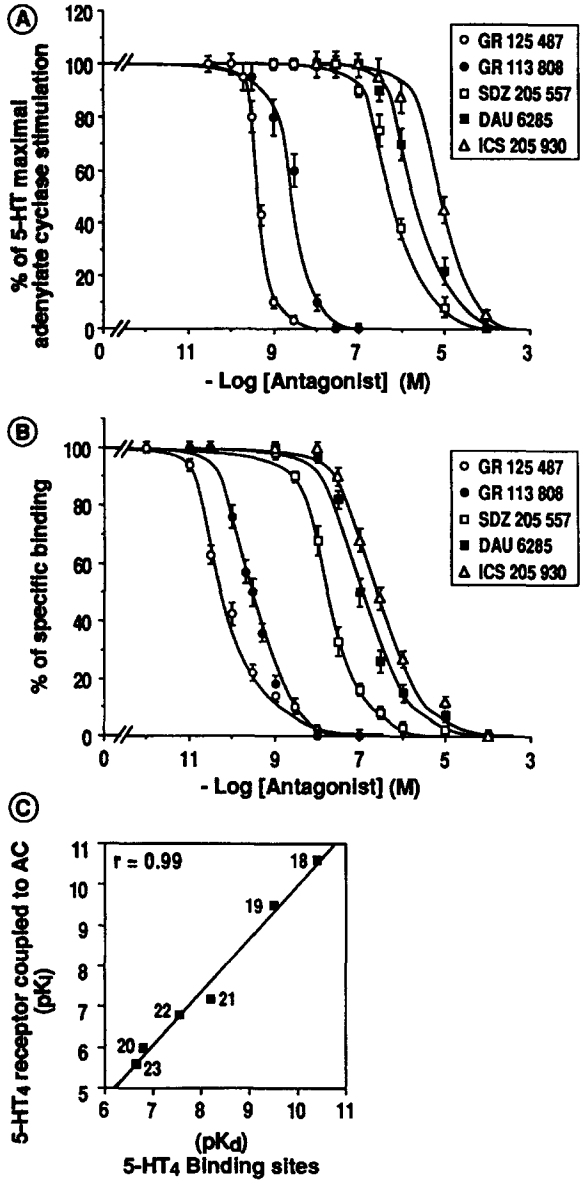

Fig. 3. Relationship between the pharmacological profiles of 5-HT$_4$ receptor antagonists that stimulate cAMP production in colliculi neurons and [^{3}H]GR 113808 binding sites in membranes of colliculi neurons. Panel A represents the activity of a series of five 5-HT$_4$ antagonists on 5-HT stimulation of cAMP formation in colliculi neurons. In the presence of 1 μM of 5-HT, neuronal cells were exposed to increasing concentrations of each antagonist and conversion of [^{3}H]ATP to [^{3}H]cAMP was determined after 5 min at 37°C. The basal conversion was 1.2 ± 0.15% (n = 16) and 5-HT-stimulated conversion was 2.5 ± 0.28% (n = 10). The results are expressed as the percentage of residual stimulation relative to the stimulatory action of 1 μM 5-HT taken as 100%. Data are the means ± S.E.M. from three separate experiments performed in duplicate. Panel B represents competition experiments carried out in colliculi neuronal membranes on 0.2 nM [^{3}H]GR 113808 binding with five 5-HT$_4$ receptor antagonists. Results are expressed as a percentage of the specific binding in the absence of a competing agent and are the means ± S.E.M. of four separate experiments. Panel C represents correlation between the affinities of six antagonists for [^{3}H]GR 113808 binding sites and the affinities of the same drugs for inhibiting 5-HT-induced cAMP production in colliculi neuronal cells. The pK_d values of 6 compounds (X axis) for [^{3}H]GR 113808 binding sites are correlated with the pK_i values for antagonists (Y axis) that block 5-HT$_4$ receptor-mediated cAMP production. All the values are taken from Table 1.

5-HT, were from nanomolar to submicromolar ranges. Table 1 provides the dissociation constant (pK_d) of 5-HT_4 receptor agonists. These values were compared to the affinities of the agonists in stimulating cAMP formation (pEC_{50}). As seen in Fig. 2C, significant correlation ($r = 0.87$, $P < 0.01$) was found between pEC_{50} and pK_d of a series of 15 agonists. An even better correlation was found between pK_i and pK_d ($r = 0.99$, $P < 0.01$) (Fig. 3C) for the most representative antagonists used to inhibit 5-HT stimulation of cAMP production (Fig. 3A) and to compete with [^{3}H]GR 113808 binding (Fig. 3B). For all the drugs tested, the Hill coefficient of the binding competition curves was not different from 1 except for 5-MeOT and metoclopramide which was less than 1. The significance of this finding is not clear.

In order to verify the similarities between [^{3}H]GR 113808 binding sites present in cell membranes of mouse embryonic colliculi neurons with those in adult guinea-pig and human striatum, we compared the pK_d of a series of 5-HT_4 ligands that compete for [^{3}H]GR 113808 binding sites in these three different preparations (Table 2; data taken from Grossman et al., 1993; Waeber et al., 1993 for pKd values in guinea-pig and human striatum, respectively). As seen in Fig. 4, significant correlations were also obtained.

The specificity of [^{3}H]GR 113808 binding sites was further tested by verifying that several potent agonists and antagonists at other 5-HT receptors, such as 5-HT_1 and 5-HT_2 receptor ligands (8-OH-DPAT, RU 24969, methiothepin and ketanserine), a benzamide acting on dopaminergic receptors (sulpiride), and several tricyclic antipsychotics acting at 5-HT_6 receptor binding sites (clozapine, amoxapine, amitriptyline), were essentially inactive.

3.3. Evolution of [^{3}H]GR 113808 binding in colliculi neuronal membranes during desensitization

In the following experiments, the time course of desensitization of 5-HT_4 receptor-mediated stimulation of cAMP was compared to the time course of the decrease in the density of [^{3}H]GR 113808 binding sites during different incubation periods with 5-HT (from 2 min to 24 h) (Fig. 5).

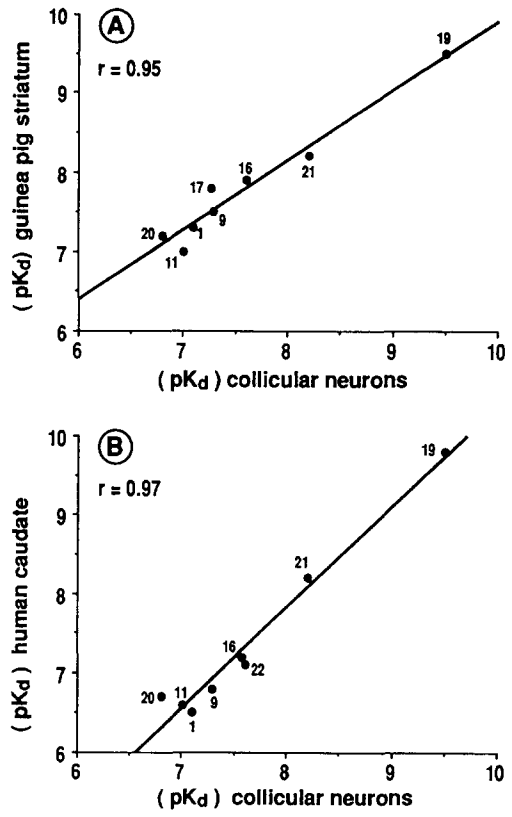

Fig. 4. Correlation between the affinities of a series of 5-HT_4 ligands for [^{3}H]GR 113808 binding sites: (A) in guinea-pig striatum (pK_d values taken from Grossman et al. (1993)) (B) in human caudate (pK_d values taken from Waeber et al. (1993)) versus their affinities in cell membranes of colliculi neurons (Table 2).

The rate of accumulation of cAMP stimulated by 5-HT decreased by 50% after a 5 min exposure period with 5-HT as reported in Fig. 5 and previously by Ansanay et al. (1992). During this period (5 min), no significant decrease in the amount of [^{3}H]GR 113808 binding sites

Table 2
Affinities of various compounds that compete for 0.2 nM [^{3}H]GR 113808 in cell membranes of colliculi neurons (Table 1), human caudate (Waeber et al., 1993) and guinea-pig striatum (Grossman et al., 1993; Waeber et al., 1993)

Compounds	No.	Colliculi neurons		Human caudate		Guinea-pig striatum
		pK_d	±(S.E.M.)	pK_d	±(S.E.M.)	pK_d [a]
GR 113808	19	9.5	(0.20)	9.8	(0.10)	9.5
SDZ 205557	21	8.2	(0.22)	8.2	(0.20)	8.2
BIMU 8	16	7.6	(0.18)	7.1	(0.10)	7.9
DAU 6285	22	7.57	(0.15)	7.2	(0.10)	N.D.
Cisapride	9	7.29	(0.24)	6.8	(0.30)	7.5
BIMU 1	17	7.27	(0.15)		N.D.	7.8
5-HT	1	7. 1	(0.11)	6. 5	(0.20)	7.3
Renzapride	11	7.01	(0.12)	6.6	(0.20)	7
ICS 205930	20	6.8	(0.30)	6.7	(0.30)	7.2
5-MeOT	2	6.28 [b]	(0.17)	6.3	(0.10)	6.5

[a] ± S.E.M.(≤ 0.2). [b] Cf. Table 1.

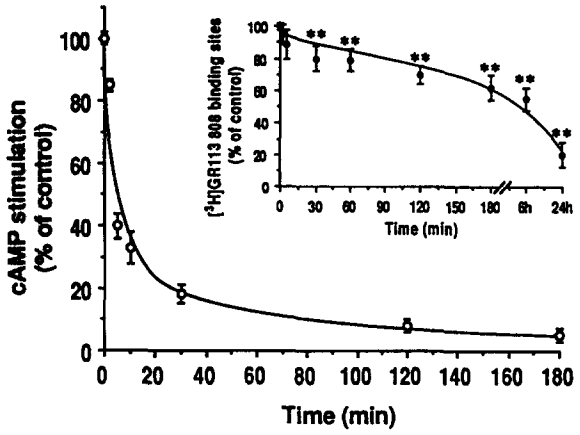

Fig. 5. Comparison between the time course of desensitization of 5-HT_4 receptors and the time course of down-regulation of [^{3}H]GR 113808 binding sites in colliculi neurons. Neurons were preincubated with 5-HT (10 μM) for the indicated time (0–24 h desensitization periods). After three washes with culture medium, cAMP accumulation was started by adding culture medium containing IBMX (0.75 mM) plus forskolin (0.1 μM) plus 5-HT (10 μM) and measured for 5 min (cAMP accumulation period). Results are expressed as a percentage of residual stimulation relative to the maximal stimulation induced by 5-HT (10 μM) taken as 100%. The basal conversion of [^{3}H]ATP to [^{3}H]cAMP was 0.8 ± 0.1%. 5-HT (10 μM)-stimulated conversion was 2.4 ± 0.35. The results are the means $\pm$ S.E.M. of three separate experiments performed in duplicate. Inset: effect of exposure to 5-HT (10 μM) on the density of [^{3}H]GR 113808 binding sites in cell membranes of colliculi neurons: neurons in culture treated with 5-HT (10 μM) for various periods of time ranging from 0 to 24 h, were washed 3 times with phosphate-buffered saline and harvested. Membranes were then prepared for binding assays as described in Materials and Methods. The density of 5-HT_4 receptors and the affinity of the receptors for [^{3}H]GR 113808 binding sites was determined by Scatchard analysis of the specific binding of [^{3}H]GR 113808 (0–0.7 nM). Non-specific binding was defined using 50 μM 5-HT. B_{max} values were expressed as percentage of the density of [^{3}H]GR 113808 binding sites in cell membranes of colliculi neurons. Without any treatment with 5-HT (control), B_{max} was 75 ± 23 fmol/mg protein. This value was taken as 100%. Data are expressed as means $\pm$ S.E.M. ($n = 5$). * * Values significantly different from those obtained without any treatment ($P < 0.05$).

was observed ($P > 0.05$) (Fig. 5, inset). After a 10 min exposure with 5-HT, the kinetic of decrease in cAMP stimulation was slower, leading to a complete loss of the response after 3 h. Clearly, the functional desensitization proceed in two phases. Only the second phase was accompanied by a reduction in [^{3}H]GR 113808 binding sites (Fig. 5, inset). This reduction was only significant after 30 min ($P < 0.05$). Yet a significant percentage of [^{3}H]GR 113808 binding sites (about 20%) remained even after exposure to 5-HT for 24h. (Fig. 5, inset). Regardless of the time of exposure with 5-HT, Scatchard analysis of the binding showed no change in the K_d for [^{3}H]GR 113808 (data not shown),.

4. Discussion

The availability of the potent and selective radioligand antagonist, [^{3}H]GR113808 (Grossman et al., 1993), has provided a new method to study 5-HT_4 receptors. The aim of the present report was to analyze the pharmacological profile of the [^{3}H]GR 113808 binding sites in cell membranes of mouse colliculi neurons, a reference model in which the 5-HT_4 receptors were described for the first time six years ago in functional studies (Dumuis et al., 1988).

The main conclusion is that [^{3}H]GR 113808 binding sites in cell membranes of colliculi neurons possess a pharmacological profile identical to that of 5-HT_4 receptors in the same model. Table 1 summarizes the affinities of agonists and antagonists structurally related to the three main chemical classes. A highly significant correlation was obtained when we compared the affinities of the compounds for the 5-HT_4 receptor-stimulating adenylyl cyclase activity and their affinities for [^{3}H]GR 113808 binding sites. For the indole class, 5-HT is the most potent agonist, followed by 5-MeOT, RU 28253 and 5-CT (5-carboxamidotryptamine). In colliculi neurons, as in human striatum and in porcine caudate nucleus, the indole compounds exhibited lower affinities in binding assay than in functional studies (see Fig. 3 and Table 1) (Grossman et al., 1993; Waeber et al., 1993; Schiavi et al., 1994). In contrast, benzamide and benzamidazolone potency values were somewhat higher in binding assays than in adenylyl cyclase assays (Table 1). For the benzamide derivatives, only the 2-methoxy-4-amino-5-chloro-substituted benzamides were active. To date cisapride and its stucturally related analogue RN 76186 are the most potent and selective 5-HT_4 receptor agonists among benzamide derivatives tested in colliculi neurons and in rat oesophagus (Briejer et al., 1993). They both exhibit a very low affinity for 5-HT_3 receptors. ML 10302, a new 2-methoxy-4-amino-5-chloro-substituted benzoate derivative is one of the most potent and selective, but partial 5-HT_4 receptor agonists in the oesophagus preparation (Langlois et al., 1994). However, it has to be noted that ML 10302 is an antagonist in colliculi neurons (unpublished results).

Of the antagonists belonging to one of the three main classes (indoles, benzamides and benzimidazolones), the rank order of potencies was similar when analyzed either in binding or in functional studies (Fig. 3 and Table 1). However, the much higher affinity of SDZ 205557 and DAU 6285 for [^{3}H]GR 113808 binding sites than for the 5-HT_4 receptor remains to be explained. A similar high affinity of these drugs for [^{3}H]GR 113808 binding sites has been reported in brain from different species (Grossman et al., 1993; Waeber et al., 1993; Schiavi et al., 1994). We found similar affinities for a series of drugs competing for [^{3}H]GR 113808 binding sites in cell membranes of mouse colliculi neurons and in human and guinea-pig striatum (Fig. 4). However, the intrinsic activities of some compounds, relative to 5-HT, specifically benzamide and benzimidazolone derivatives, seem to vary between species and tissues. Some of these discrepancies have been reported and discussed previously (Bockaert et al., 1992; Clarke and Bockaert, 1993). For example, in piglet and

human atria (Kaumann, 1990; Kaumann et al., 1991), in human myocytes (Ouadid et al., 1992) and in guinea-pig hippocampal membranes (Bockaert et al., 1990), the benzamides are weak partial agonists. Cisapride, one of the most potent 5-HT_4 receptor agonists in several tissues and species (Dumuis et al., 1989), has been reported to be a relatively weak agonist in human myocytes (Ouadid et al., 1992) and in guinea-pig ileum contraction (Craig and Clarke, 1990). Similarly, SC 53116, one of the most potent and selective 5-HT_4 receptor agonists in colliculi neurons and in rat oesophagus, was only a very weak partial agonist in human myocytes (Gullikson et al., unpublished results). 5-MeOT is as potent as 5-HT in most preparations but has been reported to be a weak agonist on rat oesophagus and guinea-pig ileum (Reeves et al., 1991). These observations may be tentatively explained either by differences in coupling efficiencies and 5-HT_4 receptor number or by the possible expression of different subtypes of 5-HT_4 receptors in these preparations. Recognition of the primary structure of the 5-HT_4 receptors present in central and peripheral tissues of different species is required to answer to this question.

Gerald et al. (1995) have recently isolated two splice variants of the 5-HT_4 receptor: 5-HT_{4L} and 5-HT_{4S}. The transcripts of the former variant are expressed throughout the brain except in cerebellum, whereas those of the latter are restricted to the striatum. Both isoforms are also present in peripheral tissues such as ileum, colon and bladder; only the 5-HT_{4S} isoform is present in heart atrium.

Another aim of the present study was to determine whether activation of 5-HT_4 receptors leads to a down-regulation of [^{3}H]GR 113808 binding sites. Until now, this work has been hampered by the lack of suitable 5-HT_4 radioligands.

We have previously described in detail (Ansanay et al., 1992) that exposure of colliculi neuronal cells to 5-HT results in a rapid homologous 5-HT_4 receptor desensitization, a cAMP-independent process leading to a 50% decrease in functional response after a 5 min exposure to 5-HT. Continuing the exposure to 5-HT results in a slower desensitization phase leading to a complete loss of 5-HT_4 receptor-mediated response after 3 h. The biphasic kinetics of desensitization was confirmed in the present study (Fig. 5). [^{3}H]GR 113808 binding studies demonstrated that the rapid desensitization of the 5-HT_4 receptor-stimulated adenylyl cyclase activity was not accompanied by any decrease in the number of binding sites (Fig. 5, insert). We can conclude that the first rapid phase of homologous desensitization was due to an alteration in the coupling between the receptor and Gs, probably resulting from a phosphorylation of the receptors similar to that described for other G protein-coupled receptors (Lefkowitz, 1993; Lefkowitz et al., 1993). The second phase of desensitization was accompanied by a loss of binding sites (down-regulation) (Fig. 5B). As observed for other receptors (Ivins and Molinoff, 1991), a pool of approximately 20% of the [^{3}H]GR 113808 binding sites was conserved even after 24 h of exposure to 5-HT_4 receptor agonists.

Desensitization of the 5-HT_4 receptor has been studied in several tissues. In guinea-pig ileum, desensitization of 5-HT_4 receptors has been used to eliminate these receptors from the preparation in order to study 5-HT_3 receptor in isolation (Craig et al., 1990). In a recent study, we demonstrated that 5-HT_4 receptors expressed in oesophagus, a non-neuronal tissue, undergo a desensitization process comparable in intensity and velocity to that described in colliculi neurons (Rondé et al., 1995). Thus, the rapid homologous desensitization of 5-HT_4 receptors observed is not restricted to neurons (Dohlman et al., 1991). However, human atria 5-HT_4 receptors appear to desensitize to a lesser extent than 5-HT_4 receptors in mouse embryonic colliculi neurons and rat oesophagus (Kaumann et al., 1991).

Again, it will be very interesting to examine whether these differences in receptor desensitization correspond to different subtypes of 5-HT_4 receptors expressed in these tissues, different coupling mechanisms between the receptor and G proteins or different involvements of receptor kinases and arrestins (Lohse et al., 1990; Dolph et al., 1993),

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