Naunyn-Schmiedeberg's Arch Pharmacol (1989) 340:403–410

Naunyn-Schmiedeberg's
Archives of
Pharmacology

The gastrointestinal prokinetic benzamide derivatives are agonists at the non-classical 5-HT receptor (5-HT_4) positively coupled to adenylate cyclase in neurons

Aline Dumuis*, Michèle Sebben, and Joël Bockaert

Centre CNRS-INSERM de Pharmacologie-Endocrinologie, Rue de la Cardonille, F-34094 Montpellier Cedex 2, France

Summary. We have previously shown that a non-classical 5-hydroxytryptamine (5-HT_4) receptor mediates the stimulation of adenylate cyclase activity in mouse embryo colliculi neurons in primary culture. The pharmacological characteristics of this receptor exclude the possibility that it belongs to the known 5-HT_1, 5-HT_2 or 5-HT_3 receptor types. Here we report that this 5-HT receptor can be stimulated by 4-amino-5-chloro-2-methoxy substituted benzamide derivatives. All these compounds have been reported to be potent stimulants of gastrointestinal motility and some of them are 5-HT_3 receptor antagonists. The rank order of potency of these substituted benzamide derivatives in stimulating cAMP formation was: cisapride > BRL 24924 > 5-HT > zacopride > BRL 20627 > metoclopramide. The non-additivity of benzamide and 5-HT activities suggests that 5-HT and the substituted benzamide derivatives act on the same receptor. Only ICS 205930, a recognized 5-HT_3 receptor antagonist, competitively antagonized the stimulatory effect of cisapride, zacopride and BRL 24924. However, its pK_i (6–6.3) for this new receptor was very different from its pK_i for 5-HT_3 receptors (pK_i = 8–10). Other selective 5-HT_3 receptor antagonists with an indole group (BRL 43694 and GR 38032F), with a benzoate group (cocaïne, MDL 72222) or with a piperazine group (quipazine) were ineffective in reversing the stimulatory effect of benzamide derivatives. Exposure of neuronal cells to potent agonists at this receptor such as BRL 24924 rapidly reduces its capacity to stimulate cAMP production. For example, a preincubation of 10 min with BRL 24924 (100 μmol/l) reduced by 42% the ability of 5-HT to stimulate cAMP production. Cross-desensitization occurs between the effects of 5-HT and benzamides. The unique pharmacology of these non-classical 5-HT receptors that we propose to call 5-HT_4 is very close and even identical to the pharmacology of the high affinity 5-HT receptors involved in the indirect stimulation of smooth muscle in the guinea pig ileum. These receptors are different from the 5-HT_3 receptors also present in guinea pig ileum.

Key words: 5-HT_4 receptor – Substituted benzamides – cAMP stimulation – Colliculi neurons – Gastrointestinal motility

Introduction

Multiple radioligand binding studies as well as biochemical, physiological and behavioural studies have defined three main 5-HT receptor types, named 5-HT_1, 5-HT_2 and 5-HT_3 both in the periphery and in the central nervous system (CNS) (Peroutka 1988).

It is now well established that 5-HT_1 receptors comprise at least four subtypes: 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C} and 5-HT_{1D} (Pedigo et al. 1981; Gozlan et al. 1983; Hoyer et al. 1985; Pazos et al. 1984; Heuring and Peroutka 1987; Waeber et al. 1988a, b). Three of them, 5-HT_{1A}, 5-HT_{1B} and 5-HT_{1D} are negatively coupled to adenylate cyclase (Weiss et al. 1986; De Vivo and Maayani 1986; Bockaert et al. 1987; Dumuis et al. 1988a; Bouhelal et al. 1988; Hoyer and Schoeffter 1988; Schoeffter et al. 1988). 5-HT_{1A} receptors may also stimulate this enzyme (Markstein et al. 1986; Shenker et al. 1987; Dumuis et al. 1988 b) or be coupled to the K^+ channel (Andrade et al. 1986).

The 5-HT_{1C}, like 5-HT_2 receptors (Leysen et al. 1982) stimulate phospholipase C to trigger the inositol phosphate-diacylglycerol system (Conn et al. 1986; Kendall and Nahorski 1985; Conn and Sanders-Bush 1985; Chaffoy de Courcelles et al. 1985; Doyle et al. 1986; Cory et al. 1987). 5-HT_1 and 5-HT_2 receptors are therefore all coupled to their effectors by GTP binding proteins and are likely to have similar structures. Indeed, 5-HT_{1A}, 5-HT_{1C} and 5-HT_2 receptors which have recently been cloned have the classical seven transmembrane structure of the GTP binding protein linked receptors (Fargin et al. 1988; Lübbert et al. 1987; Pritchett et al. 1988; Julius et al. 1988).

5-HT_3 receptors defined first in the peripheral nervous system by functional experiments have recently been labelled in the CNS (Kilpatrick et al. 1987; Peroutka and Hamik 1988; Watling et al. 1988a, b; Waeber et al. 1988c). Several subtypes of 5-HT_3 receptors may exist (Richardson et al. 1985; Bradley et al. 1986). However, there are several responses both in the CNS (Bockaert et al. 1981; Shenker et al. 1987; Andrade and Nicoll 1987) and in the periphery (Sanger 1987; Buchheit et al. 1985; Mawe et al. 1986; Branchek et al. 1988; Craig and Clarke 1989), which cannot be recognized as being mediated by classical 5-HT_1, 5-HT_2 and 5-HT_3 receptors. Such receptors have been termed "orphan receptors" (Van Hueven-Nolsen 1988). In particular, there remains some doubt as to the exact relationship between the excitatory receptors of the enteric neurons defined by Gaddum and Picarelli as the M receptor (Gaddum and Picarelli 1957) and the 5-HT_3 receptors (Richardson et al. 1985; Kilpatrick et al. 1987; Fozard 1987; Hoyer and Neijt 1987, 1988; Peroutka and Hamik 1988; Richardson and Buchheit 1988; Waeber et al. 1988c; Neijt et al. 1988; Barnes et al. 1988). Thus, it is certain that other types or subtypes of 5-HT receptors do exist.

Send offprint requests to A. Dumuis at the above address

We have recently described a non-classical 5-HT receptor that we have designated 5-HT_4 (Dumuis et al. 1988b). These receptors mediate stimulation of adenylate cyclase activity both in mouse embryo colliculi neurons and in guinea pig hippocampal membranes. Classical 5-HT_1 receptor agonists are inactive or weakly active at 5-HT_4 receptors and no 5-HT antagonists are able to block them, except ICS 205930, which did so with low potency ($pK_i = 6$). In a subsequent study, we have shown that two substituted benzamide derivatives, BRL24924 and metoclopramide, are agonists at these 5-HT receptors (Dumuis et al. 1989).

In this report, we present a study on the effects of 4-amino-5-chloro-2-methoxy substituted benzamide derivatives on the non-classical 5-HT_4 receptor of the CNS and discuss the possibility that these receptors are similar or identical to the high affinity 5-HT receptors involved in the prokinetic actions of 5-HT and benzamides on guinea pig ileum (Sanger 1987; Craig and Clarke 1989).

Materials and methods

Culture of mouse embryo colliculi neurons. Briefly, 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 previously treated 12-well Costar tissue culture dishes (Dumuis et al. 1988b). Cultures were maintained at 37° C in a humidified atmosphere in 6% CO_2/94% air. These culture conditions have already been described in detail for striatal and cortical neurons (Weiss et al. 1986).

Cyclic AMP formation. Intracellular cyclic AMP levels were determined by measuring the conversion of the [^{3}H]-adenine nucleotide precursor: [^{3}H]-ATP to [^{3}H]-cAMP.

On the sixth day of culture and before each experiment, neurons were incubated at 37° C for 2 h with culture media containing 2 μCi/ml [^{3}H]-adenine (24 Ci/mmol) (Amersham, UK). After 2 h, the cultures were washed and incubated with isobutyl-methylxanthine (IBMX) 0.75 mmol/l and forskolin 0.1 μmol/l and test agents (all in culture media) in a volume of 1 ml for 10 min at 37° C. The reaction was stopped by aspiration of the media and addition of 1 ml ice-cold 5% trichloracetic acid. Cells were loosened with the aid of a rubber scraper and 100 μl of 5 mmol/l ATP + 5 mmol/l cAMP was added to the mixture.

Cellular protein was centrifuged at $5{,}000 \times g$ and the supernatant was eluted through sequential chromatography on Dowex and alumina columns which permitted the separation of [^{3}H]-ATP and [^{3}H]-cAMP. We have previously shown that in neuronal cultures, 0.1μmol/l forskolin does not modify basal cyclic AMP concentrations but increases neurotransmitter efficacy in cyclic AMP production, whereas potency remains unaffected (see Fig. 1 in article by Weiss et al. 1985).

Data analysis. The mean values of at least three experiments performed in duplicate have been given ± standard errors (vertical bars). EC_{50} refers to the agonist concentrations yielding 50% of the maximal activation determined directly on each concentration response curve, pEC_{50} is the negative logarithm of EC_{50}. The K_i values of antagonists were calculated from the concentration of the drug reversing the stimulation obtained with agonists (1 μmol/l) by 50%, using the Cheng-Prusoff equation (1973) or by using the method of Arunlakshana and Schild (1959). pK_i is the negative logarithm of K_i.

Drugs. The following drugs were generously donated: BRL24924: [(±)-endo]-4-amino-5-chloro-2-methoxy-N-(1-azabicyclo-[3.3.1]-non-4-yl) benzamide mono-hydro-chloride. BRL20627: [2-α,6β,9aα)-(±)-4-amino-5-chloro-2-methoxy-N-(octa-hydro-6-methyl-2-H-quinolizin)-benzamide HCl]. BRL43694: endo-N-(9-methyl-9-azabicyclo-[3.3.1]-non-3-yl)-1-methyl-1H-indazol-3-carboxamide. BRL34915: and ± 6-cyano-3,4-dihydro-2,2-dimethyl-trans-4-(2-oxo-1-pyrrolidyl)-2H-1-benzo-pyran-3-ol (Sanger, GJ, Beecham Pharmaceuticals, Harlow, UK). Cisapride: cis-4-amino-5-chloro-N[1-[3-(4-fluoro-phenoxy)propyl]-3-methoxy-4-piperidinyl]-2-methoxy benzamide (Janssen Pharmaceutica, Belgium). Metoclopramide (Laboratoires Delagrange, Paris, France). Zacopride: 4-amino-N-(1-azabicyclo[2.2.2]oct-3yl)-5-chloro-2-methoxy-benzamide HCl (Laboratoires Delalande, Rueil-Malmaison, France). MDL72222: 1αH, 3α,5αH-tropan-3-yl-3,5-dichlorobenzoate (Merrell Dow Research Institute, Strasbourg, France). GR38032F: 1,2,3,9-tetrahydro-9-methyl-3[(2-methyl-1H-imidazol-1-yl)methyl]-4H-carbazol-4-one (Glaxo Research Group, Ware, Hertfordshire, UK). The following drugs were purchased: 5-HT: 5-hydroxytryptamine (Sigma Chemical Co., St. Louis, MO, USA). ICS205930: [(3α-tropanyl)-1H-indole-3-carboxylic acid ester] and isoprenaline (R.B.I., USA).

Results

Effect of 5-HT and 4-amino-5-chloro-2-methoxy substituted benzamide derivatives on cyclic AMP production in colliculi neurons. Metoclopramide, the well-known 4-amino-5-chloro-2-methoxy substituted benzamide derivative (Fig. 1) was a weak partial agonist of this receptor: $pEC_{50} = 5.34 \pm 0.37$ ($n = 3$) eliciting 44 ± 5% of the maximal 5-HT effect taken as 100% (Fig. 2). BRL20627 derived from metoclopramide by incorporating the amino ethyl side chain into a rigid ring system (Fig. 1). This gave a compound having almost the same affinity for this 5-HT receptor: $pEC_{50} = 5.49 \pm 0.23$ ($n = 3$) eliciting 60% of the maximal 5-HT effect (Fig. 2). Substitution of the condensed nitrogen-containing 2-ring system of BRL20627 by a bridged 2-ring system led to the compound BRL24924 (Fig. 1). This modification resulted in a dramatic increase in both the affinity for the 5-HT receptor ($pEC_{50} = 6.9 \pm 0.12$, $n = 4$) and efficacy (133 ± 15%, $n = 4$) of the maximal 5-HT effect.

Zacopride, a substituted benzamide (Fig. 1) reported as being a selective 5-HT_3 receptor ligand (Barnes et al. 1988) was an agonist in stimulating adenylate cyclase in colliculi ligand neurons ($pEC_{50} = 5.95 \pm 0.24$, $n = 4$) eliciting 144 ± 22% of the maximal 5-HT effect.

Cisapride, another 4-amino-5-chloro-2-methoxy substituted benzamide with a long and bulky side chain (Fig. 1) and which was developed as a gastrointestinal stimulatory agent (Schuurkes et al. 1985, 1987), appeared to be the most effective agonist at this 5-HT receptor ($pEC_{50} = 7.14 \pm 0.11$, $n = 6$), eliciting 142 ± 17% of the maximal 5-HT effect (Fig. 2).

In order to verify that the compounds of the benzamide family (Fig. 1) interact with the same non-classical 5-HT receptor which mediates the 5-HT effects on this system, we

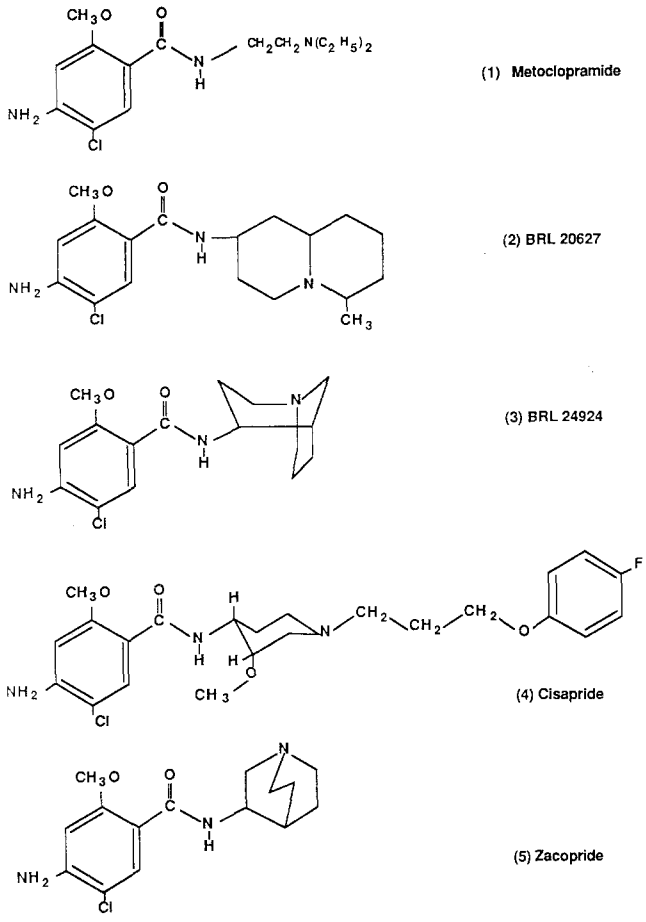

Fig. 1. The structural formulae of five 4-amino-5-chloro-2-methoxy substituted benzamide derivatives whose abilities to stimulate cAMP formation in mouse embryo colliculi neurons were assessed

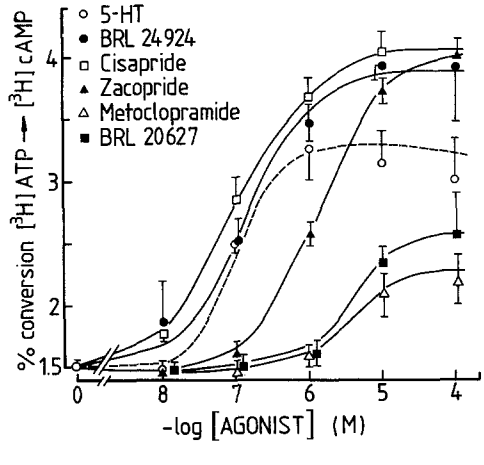

Fig. 2. Stimulation of cAMP formation in mouse embryo colliculi neurons by 5-HT and by five substituted benzamide derivatives: cisapride, BRL24924, zacopride, BRL20627 and metoclopramide. Cells were incubated with a low concentration of forskolin (0.1 μmol/l) and increasing concentrations of each agonist. Conversion of [^{3}H]-ATP to [^{3}H]-cAMP was determined after 10 min at 37° C as described under Materials and methods. In the absence of agonists, the percentage conversion was 1.5 ± 0.13%. The results are expressed as the percentage of the conversion of [^{3}H]-ATP to [^{3}H]-cAMP ± standard error of at least three separate experiments each performed in duplicate

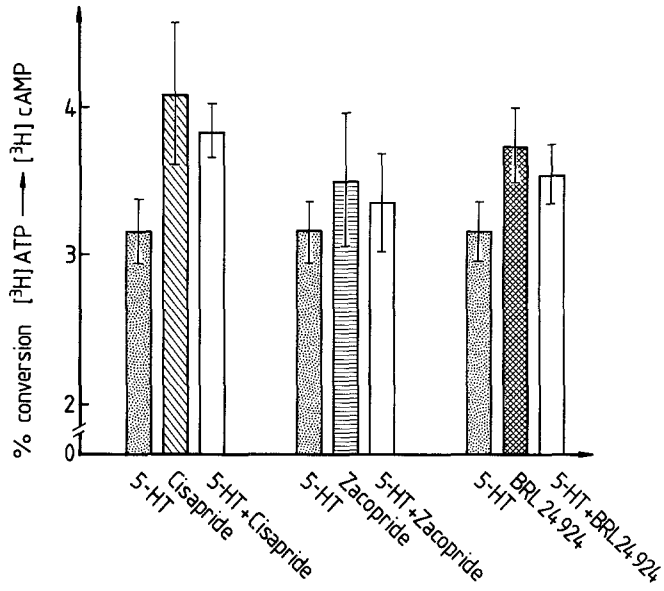

Fig. 3. Effect of substituted benzamide derivatives (cisapride, zacopride or BRL24924) on 5-HT stimulated formation of cAMP in mouse embryo colliculi neurons. The effects of cisapride, zacopride or BRL24924 (10 μmol/l each) on cAMP formation were determined independently and in the presence of 5-HT (10 μmol/l). Substituted benzamide derivatives were added simultaneously with 5-HT and the responses were determined after 10 min as the percentage conversion of [^{3}H]-ATP to [^{3}H]-cAMP ± standard errors (SEM) of at least three separate experiments each performed in duplicate

studied the additivity of the effect of 5-HT with those of benzamides on cAMP production.

The percent conversion of [^{3}H]-ATP to [^{3}H]-cAMP in the presence of 10 μmol/l 5-HT and cisapride were 3.15 ± 0.17% and 4.07 ± 0.48%, ($n = 5$), respectively. In the presence of the two drugs, the percent conversion was 3.8 ± 0.19%, ($n = 5$). Similar results were obtained with the two other potent agonists, zacopride and BRL24924 (Fig. 3). Therefore, no additivity of the effects of these benzamides with those of 5-HT was observed. In contrast, a total additivity was observed when we tested the effects of 10 μmol/l isoprenaline (a β-adrenoceptor agonist) with those of 10 μmol/l 5-HT. The percent stimulation over the basal activity level was 123 ± 19% and 126 ± 14%, ($n = 3$), in the presence of 10 μmol/l 5-HT and 10 μmol/l isoprenaline respectively, and was 221 ± 52% when 10 μmol/l 5-HT and 10 μmol/l isoprenaline were incubated simultaneously.

Effect of ICS205930, on benzamide derivative-induced stimulation of cAMP. We have previously reported that ICS 205930, a selective 5-HT$_3$ receptor antagonist (Richardson et al. 1985), competitively inhibited 5-HT stimulation of cAMP production (Dumuis et al. 1988b). This 5-HT$_3$ receptor antagonist was also able to reverse completely the stimulatory effect of 10 μmol/l zacopride or 1 μmol/l BRL24924 as shown in Fig. 4. The pK_i values of ICS205930 for blocking the cisapride, zacopride and BRL24924 effects were 6.14 ± 0.07 ($n = 4$), 6.23 ± 0.12 ($n = 4$) and 6.27 ± 0.12 ($n = 4$), respectively. The ability of four concentrations of ICS205930 (10, 30, 60 and 100 μmol/l) to shift the BRL24924 concentration response curve to the right is shown in Fig. 5. ICS205930 competitively inhibited the stimulatory effect of BRL24924 as indicated by the Schild

plot (slope = 1); the pA_2 value was 6.30 ± 0.06 ($n = 5$).

Other selective 5-HT_3 receptor antagonists having an indole group (BRL43694, GR38032F), a benzoate group (cocaine and MDL72222) or a piperazine group (quipazine) were ineffective in blocking 5-HT stimulating cAMP production (Table 1).

BRL34915 reported to be a stimulant of K^+ conductance in cardiac muscle (Scholtysik 1987) was inactive both as an agonist or antagonist at this non-classical receptor (Table 1).

Rapid desensitisation of the non-classical 5-HT receptor stimulating cAMP production in colliculi neurons. We have seen (Fig. 3) that the BRL24924 and 5-HT effects were not additive when the two drugs were added during the same incubation period. However, when colliculi neurons were preincubated with BRL24924 for 10 min at two different concentrations (10 µmol/l and 100 µmol/l) before starting the 5-HT (1 µmol/l) stimulated accumulation of cAMP, by

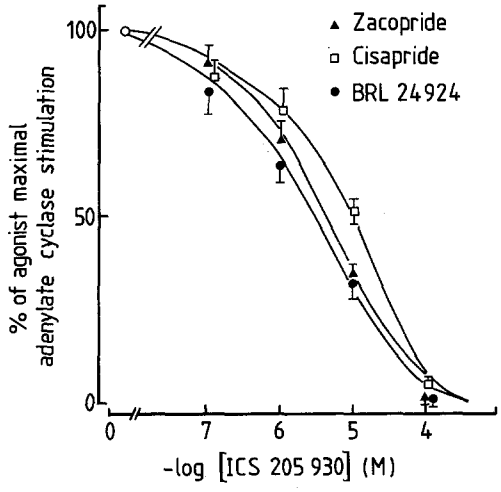

Fig. 4. Effect of ICS205930 on BRL24924, cisapride, and zacopride stimulation of cAMP formation in mouse embryo colliculi neurons. In the presence of either 1 µmol/l BRL24924, or 1 µmol/l cisapride, or 10 µmol/l zacopride plus 0.1 µmol/l forskolin, neuronal cells were exposed to increasing concentrations of ICS205930. Expressed as a percent of residual stimulation relative to the maximum stimulatory action of each agonist taken as 100%. In the presence of BRL 24924, cisapride and zacopride, the percentage conversion of [^{3}H]-ATP to [^{3}H]-cAMP was $3.4 \pm 0.18\%$, $3.06 \pm 0.15\%$, and $3.7 \pm 0.1\%$ ($n = 4$) respectively. In the absence of agonists, it was $1.5 \pm 0.1\%$ ($n = 4$). The results are the means $\pm$ SEM of four separate experiments performed in duplicate

Table 1. Activity of a series of substituted benzamide agonists and selective 5-HT_3 receptor antagonists on the 5-HT_4 receptors in mouse embryo colliculi neurons

Agonists	pEC_{50}	E%	*n*
5-HT	7.03 ± 0.09	100	6
Cisapride	7.14 ± 0.11	142 ± 17	6
BRL24924	6.90 ± 0.12	133 ± 15	4
Zacopride	5.95 ± 0.24	144 ± 22	4
BRL20627	5.49 ± 0.23	60 ± 7	3
Metoclopramide	5.34 ± 0.37	44 ± 5	3
Antagonists	**pK_i**		***n***
ICS205930 (5-HT: 1 µmol/l)	6.01 ± 0.23		4
ICS205930 (Cisapride: 1 µmol/l)	6.14 ± 0.07		4
ICS205930 (BRL24924: 1µmol/l)	6.27 ± 0.14		4
ICS205930 (Zacopride: 10 µmol/l)	6.23 ± 0.12		4
BRL43694	> 5		3
BR38032F	> 5		3
MDL72222	> 5.3		3
Cocaine	inactive		3
Quipazine	inactive		3
BRL34915	inactive		3

The effects of *agonists* on adenylate cyclase activity were determined as described under Materials and methods. Data are expressed as pEC_{50}, means $\pm$ standard errors. The efficacy (E) of the agonist is the maximal stimulatory effect on adenylate cyclase activity as a percentage of the maximal stimulatory effect of 5-HT (taken as 100%). The effects of *antagonists* determined as described under Materials and methods are expressed as pK_i, means $\pm$ standard errors. The pK_i values were calculated, for ICS205930, from the concentration of the antagonist reversing the stimulation obtained with different agonists (5-HT, cisapride, BRL24924, zacopride) by 50%. The effects of other antagonists were tested only on 5-HT stimulation (1 µmol/l)

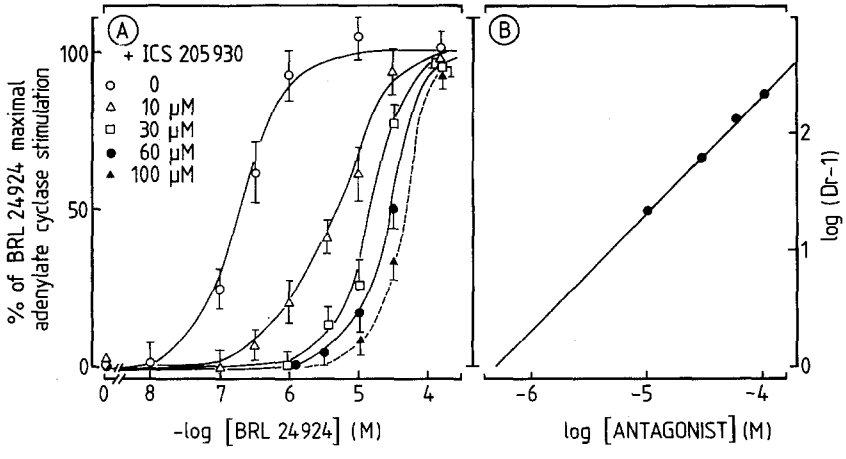

Fig. 5. Antagonism of BRL24924 induced stimulation of cAMP formation by ICS 205930 in mouse embryo colliculi neurons in primary culture. (**A**) BRL24924 concentration effect curves are represented alone and in the presence of 10, 30, 60 and 100 µmol/l ICS205930. Results are expressed as a percent of stimulation relative to the maximum stimulatory action of BRL24924 taken as 100%. In the absence of BRL24924, the percentage conversion of [^{3}H]-ATP to [^{3}H]-cAMP was $1.4 \pm 0.2\%$ ($n = 4$) and $3.5 \pm 0.15\%$ ($n = 4$) at the maximum stimulatory action of BRL24924, respectively. Each point is the means $\pm$ SEM of four separate experiments each performed in duplicate. (**B**) Schild plot analysis of the experiments represented in (**A**)

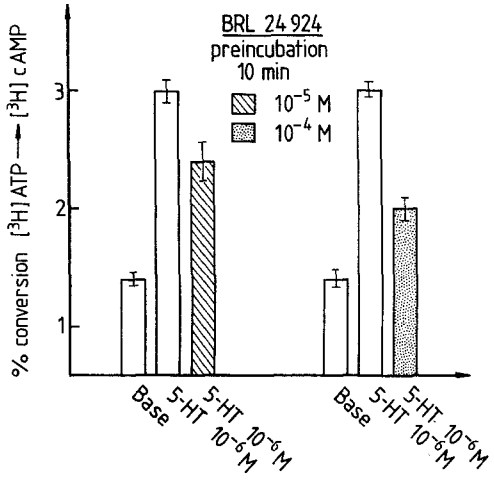

Fig. 6. Effect of BRL24924 on the desensitization of non-classical 5-HT receptors stimulating cAMP production in mouse embryo colliculi neurons. Neuronal cells were incubated without or with two different concentrations of BRL24924 (10 µmol/l and 100 µmol/l) for 10 min prior to measuring cAMP accumulation. Cells were washed with culture medium and then incubated with IBMX (0.75 mmol/l) plus 0.1 µmol/l forskolin and cAMP stimulated-5-HT (1 µmol/l; see methods). Results are expressed as the percentage of the conversion of [^{3}H]-ATP to [^{3}H]-cAMP ± standard errors of at least three separate experiments each performed in duplicate

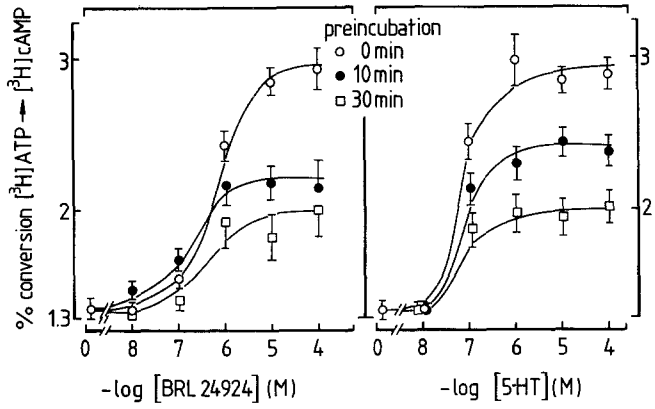

Fig. 7. Influence of the preincubation time with agonists (5-HT or BRL24924) on the desensitization of the 5-HT receptors mediating stimulation of cAMP formation in mouse embryo colliculi neurons. Neuronal cells were exposed either to increasing concentrations of BRL24924 (10^{-8} mol/l to 10^{-4} mol/l) or to increasing concentrations of 5-HT (10^{-8} mol/l to 10^{-4} mol/l) for 10 min and 30 min before measuring cAMP accumulation. Controls without preincubation with agonists were performed in each case. Neurons were then rapidly washed with culture medium and then incubated with IBMX (0.75 mmol/l) plus 0.1 µmol/l forskolin and cAMP stimulating agents BRL24924 (10^{-8} mol/l to 10^{-4} mol/l) or 5-HT (10^{-8} mol/l to 10^{-4} mol/l) for 10 min as described under Materials and methods. Results are expressed as the percentage of the conversion of [^{3}H]-ATP to [^{3}H]-cAMP ± standard errors of at least three separate experiments each performed in duplicate

adding IBMX (0.75 mmol/l), plus forskolin (0.1 µmol/l) it was observed that these treatments reduced by 24% and 42% respectively the maximal adenylate cyclase stimulation obtained (Fig. 6).

When colliculi neurons were preincubated with different concentrations of BRL24924 or 5-HT at different times before beginning the accumulation of cAMP by adding IBMX (0.75 mmol/l) plus forskolin (0.1 µmol/l), it was observed that the maximal stimulation obtained with 5-HT and BRL24924 was greatly reduced (Fig. 7), thus, 5-HT and BRL24924 both rapidly desensitized the non-classical 5-HT receptor response.

Discussion

This report confirms the existence, in the central nervous system, of a 5-HT receptor having unique pharmacological properties clearly different from those of 5-HT_1, 5-HT_2 and 5-HT_3 receptors. This receptor, which stimulates cyclic AMP production in mouse colliculi neurons in primary culture and in guinea pig hippocampal membranes is not stimulated by the classical 5-HT_{1A} receptor agonists (8-OH-DPAT or ipsapirone), has a weak affinity for 5-carboxamidotryptamine (pEC_{50} = 4.5 to 5.5) and is not inhibited by classical 5-HT_1 and 5-HT_2 receptor antagonists (Dumuis et al. 1988b).

Several data clearly indicate that the receptor does not belong to the 5-HT_3 receptor family: (a) analogues of 5-HT substituted in position 5 of the indole ring such as 5-methoxytryptamine or tryptamine itself are totally inactive at 5-HT_3 receptors (Richardson and Buchheit 1988) yet are full agonists of the non-classical 5-HT receptor described here (Dumuis et al. 1988b), (b) 2-methyl-5-HT a potent 5-HT_3 agonist is almost inactive (Dumuis et al. 1988b), (c) all 5-HT_3 receptor antagonists tested (except ICS205930) including those having an indole group (BRL43694, GR38032F), a benzoate group (cocaine and MDL72222) or a piperazine group (quipazine) were completely inactive, (d) the affinity of ICS 205930 for this non-classical receptor stimulating cyclic AMP production was far lower (pK_i = 6–6.3) than its affinity for 5-HT_3 receptors (pA_2 = 8–10) (Richardson and Buschheit 1988).

Another interesting property of this non-classical 5-HT receptor is described in detail in the present paper. Thus, all 4-amino-5-chloro-2-methoxy benzamide derivatives tested, were full or partial agonists of this receptor, including those which have been described as 5-HT_3 antagonists (BRL 20627, BRL24924, zacopride). The order of potency was: cisapride > BRL24924 > zacopride > BRL20627 > metoclopramide. The efficacies of cisapride, BRL24924 and zacopride in stimulating cyclic AMP production were higher than those of 5-HT whereas those of metoclopramide and BRL20627 were lower. The lower efficacy of 5-HT compared to those of cisapride and BRL24924 was probably not due to a competing 5-HT_1-like receptor mediated inhibition of adenylate cyclase. Indeed, 10 µmol/l of spiperone, pindolol or metergoline were unable to increase the cyclic AMP produced in the presence of 5-HT (1 µmol/l) (data not shown). Although ICS205930 and zacopride interact with these non-classical 5-HT receptors, they do not appear to label them when used in binding studies (Hoyer and Neijt 1988; Waeber et al. 1988; Barnes et al. 1988). This is probably due to their lower affinity for these non-classical 5-HT receptors than for 5-HT_3 receptors.

The unique capacity of this non-classical 5-HT receptor of neurons to be stimulated by this category of benzamide derivatives plus its absence of 5-HT_1, 5-HT_2 and 5-HT_3 pharmacological characteristics suggest that it could be use-

ful to call it 5-HT_4 as we have previously proposed (Dumuis et al. 1988b, 1989). It is likely that the 5-HT_4 receptor of the CNS also exists in the gastrointestinal system. Gaddum and Picarelli described in their classic work in guinea pig ileum that 5-HT contracts this organ by two different mechanisms (Gaddum and Picarelli 1957). One is a direct stimulation of smooth muscle through what is now known to be a 5-HT_2 receptor which was blocked by dibenzyline and was therefore called the D receptor by these authors (Gaddum and Picarelli 1957). The other is an indirect action involving excitation of intramural nerves which release neurotransmitters able to contract smooth muscle. Morphine was able to block this mechanism probably by inhibiting the release of neurotransmitters; for this reason, the 5-HT receptors involved, were called M receptors.

However, it is likely that at least two *indirect* mechanisms of 5-HT action exist in guinea pig ileum. Richardson and Buchheit (1988), Fozard (1989) as well as Sanger and Wardle (1989), clearly reported that one of these mechanisms has a high affinity for 5-HT ($pA_2 = 8$), is blocked by atropine, substance P antagonists and morphine, but not by low doses of ICS 205930 (100 nmol/l). It is sensitive to analogues of 5-HT substituted at the 5 position (Fozard 1989). All these characteristics indicate that this indirect mechanism does not involve 5-HT_3 receptors (Buchheit et al. 1985; Mawe et al. 1986; Branchek et al. 1988; Craig and Clarke 1989; Fozard 1989). The other mechanism has a lower affinity for 5-HT ($pA_2 = 6$), is more resistant to atropine, is blocked by substance P antagonists, is less sensitive than the first one to morphine and is blocked by low doses of ICS 205930 (100 nmol/l) (Buchheit et al. 1985; Sanger and Nelson 1989; Fozard 1989). It is likely that this second mechanism involves 5-HT_3 receptors (Richardson et al. 1985; Richardson and Buchheit 1988).

The question is, therefore, what is the nature of the 5-HT receptor involved in the high potency phase of the indirect neuronal stimulant response to 5-HT in guinea pig ileum? Several data indicate that they could be very close or even identical to the 5-HT_4 receptors of the CNS described here:

(1) These receptors as well as 5-HT_4 receptors are blocked by micromolar concentrations of ICS 205930 are resistant to metergoline and stimulated by 5-methoxytryptamine but not by 2-methyl-5-HT (Craig and Clarke 1989).

(2) 4-amino-5-chloro-2-methyl benzamide derivatives are agonists at the 5-HT receptors involved in cholinergic induced contraction in guinea pig ileum (Sanger 1987; Craig and Clarke 1989; Schuurkes et al. 1985, 1987). Schuurkes et al. (1987) reported the following order of potency: cisapride clebopride > metoclopramide (the effect of BRL 24924 was comparable to that of cisapride, J. A. J. Schuurkes, personal communication). This order of potency is similar to that found in the present study. Cisapride and metoclopramide have been shown to mimic 5-HT in inducing acetylcholine release in guinea pig myenteric plexus (Pfeuffer-Friederich and Kilbinger 1984).

(3) In guinea pig ileum, Sanger (1987) reported that preincubation with micromolar concentrations of 5-HT for 10 min prior to addition of BRL 24924 reduces the contractile effect of this benzamide. Similarly, Pfeuffer-Friederich and Kilbinger (1984) reported a rapid cross desensitization between 5-HT and cisapride on acetylcholine release. Such observations may be related to the data reported here concerning the rapid desensitization of 5-HT_4 receptors.

Although more data are required to propose that the non-classical 5-HT receptor of the CNS that we propose to call 5-HT_4 is identical to the high affinity 5-HT receptor involved in the indirect stimulation of the smooth muscle contraction in guinea pig ileum, it is already clear that they are pharmacologically similar.

Therefore, it appears that 5-HT_3 and 5-HT_4 receptors of enteric nerve terminals induce neurotransmitter release by two different mechanisms, an increase in cationic conductance and an increase in cAMP production, respectively. In addition to a role in guinea pig ileum contractions, 4-amino-5-chloro-2-methoxy benzamide derivatives have also been found to increase antroduodenal coordination, to block gastric relaxation induced by other neurotransmitters and to enhance colonic motility (Schuurkes et al. 1985; J. A. J. Schuurkes, personal communication). These properties support their use as gastrokinetic agents in motor dysfunctions (e.g. gastro-oesophageal efflux, gastroparesis and colonic atony). In contrast, the possible functional consequences of an action of these compounds on brain 5-HT_4 receptors remain to be discovered.

Acknowledgements. We would like to thank the gracious assistance of Mrs. A. L. Turner-Madeuf and Mrs. M. Passama for the preparation of this manuscript. Mrs. A. L. Turner-Madeuf is acknowledged for having revised the grammar. We would also like to thank Doctors G. J. Sanger, J. E. Leysen, J. A. J. Schuurkes and P. A. Janssen for interesting suggestions. This work was supported by grants from the Centre National de la Recherche Scientifique (CNRS), the Institut National de la Santé et de la Recherche Médicale (INSERM) and the Bayer and Troponwerke Laboratories (France and FRG).

References

Andrade R, Malenka RC, Nicoll RA (1986) A G protein couples serotonin and GABAB receptors to the same channels in hippocampus. Science 234:1261

Andrade R, Nicoll RA (1987) Pharmacologically distinct actions of serotonin on single pyramidal neurones of the rat hippocampus recorded in vitro. J Physiol 394:99–124

Arunlakshana O, Schild HO (1959) Some quantitative uses of drug antagonists. Br J Pharmacol 14:48–58

Barnes NM, Costall B, Naylor RJ (1988) ^{3}H-zacopride: ligand for the identification of 5-HT_3 recognition sites. J Pharmacol 40:548–551

Bockaert J, Nelson DL, Herbet A, Adrien J, Enjalbert A, Hamon M (1981) Serotonin receptors coupled with an adenylate cyclase in the rat brain: non identity with [^{3}H]-5-HT binding sites. In: Haber B, Gabay S, Issidores MR, Alvisatos SGA (eds) Serotonin-Current aspects of neurochemistry and function. Plenum Press, New York, pp 327–345

Bockaert J, Dumuis A, Bouhelal R, Sebben M, Cory RN (1987) Piperazine derivatives including the putative anxiolytic drugs, buspirone and ipsapirone are agonists at 5-HT_{1A} receptors negatively coupled with adenylate cyclase in hippocampal neurons. Naunyn-Schmiedeberg's Arch Pharmacol 335:588–592

Bouhelal R, Smounya L, Bockaert J (1988) 5-HT_{1B} receptors are negatively coupled with adenylate cyclase in rat substantia nigra. Eur J Pharmacol 151:189–196

Bradley PB, Engel G, Feniuk W, Fozard JR, Humphrey PPA, Middlemiss DN, My Lecharane EJ, Richardson BP, Saxena PR (1986) Proposals for the classification and nomenclature of functional receptors for 5-hydroxytryptamine. Neuropharmacology 25:563–576

Branchek TA, Mawe GM, Gershon MD (1988) Characterization and localization of a peripheral neural 5-hydroxytryptamine

receptor subtype (5-HT_{IP}) with a selective agonist, ^{3}H-5-hydroxyindalpine. J Neurosci 8:2582–2595

Buchheit KH, Engel G, Mutschler E, Richardson BP (1985) Study of the contractile effect of 5-hydroxytryptamine (5-HT) in the isolated longitudinal muscle strip from guinea pig ileum. Evidence for two distinct release mechanisms. Naunyn-Schmiedeberg's Arch Pharmacol 329:36–41

Chaffoy de Courcelles D, Leysen JE, De Clerck F, Van Belle H, Janssen PA (1985) Evidence that phospholipid turnover is the signal transducing system coupled to serotonin-S_2 receptor sites. J Biol Chem 260:7603–7608

Cheng Y, Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of an inhibitor that causes a 50% inhibition (I_{50}) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108

Conn J, Sanders-Bush E (1985) Serotonin-stimulated phosphoinositide turnover: Mediation by the S_2 binding site in rat cerebral cortex but not in subcortical regions. J Pharmacol Exp Ther 234:195–203

Conn J, Sanders-Bush E, Hoffman BJ, Hartig PR (1986) A unique serotonin receptor in choroid plexus is linked to phosphatidylinositol turnover. Proc Natl Acad Sci [USA] 83:4086–4088

Cory RN, Rouot B, Guillon G, Sladeczek F, Balestre MN, Bockaert J (1987) The 5-hydroxytryptamine (5-HT_2) receptor stimulates inositol phosphate formation in intact and broken WRK_1 cells: Determination of occupancy-response relationships for 5-HT agonists. J Pharmacol Exp Ther 241:258–267

Craig DA, Clarke DE (1989) 5-hydroxytryptamine and cholinergic mechanisms in guinea pig ileum. Br J Pharmacol 96:247P

De Vivo M, Maayani S (1986) Characterization of the 5-hydroxytryptamine$_{1A}$ receptor mediated inhibition of forskolin-stimulated adenylate cyclase activity in guinea pig and rat hippocampal membranes. J Pharmacol Exp Ther 238:248–253

Doyle VM, Creba JA, Rüegg UT, Hoyer D (1986) Serotonin increases the production of inositol phosphates and mobilizes calcium via the 5-HT_2 receptor in $A7r_5$ smooth muscle cells. Naunyn-Schmiedeberg's Arch Pharmacol 333:98–103

Dumuis A, Sebben M, Bockaert J (1988a) Pharmacology of 5-hydroxytryptamine$_{1A}$ receptors which inhibit cAMP production in hippocampal and cortical neurons in primary culture. Mol Pharmacol 33:178–186

Dumuis A, Bouhelal R, Sebben M, Cory R, Bockaert J (1988b) A non-classical 5-hydroxytryptamine receptor positively coupled with adenylate cyclase in the central nervous system. Mol Pharmacol 34:880–887

Dumuis A, Sebben M, Bockaert J (1989) BRL 24924: A potent agonist at a non-classical 5-HT receptor positively coupled with adenylate cyclase in colliculi neurons. Eur J Pharmacol 162:381–384

Fargin A, Raymond JR, Lohse MJ, Kobilka BK, Caron MG, Lefkowitz RJ (1988) The genomic clone G-21 which resembles a β-adrenergic receptor sequence encodes the 5-HT_{1A} receptor. Nature 335:358–360

Fozard JR (1987) 5-HT: The enigma variations. TIPS 8:501–506

Fozard JR (1989) Agonists and antagonists of 5-HT_3 receptors. In: Saxena PR (ed) Cardiovascular pharmacology of 5-hydroxytryptamine: prospective therapeutic applications. Kluwer, Dordrecht, (in press)

Gaddum JH, Picarelli ZP (1957) Two kinds of tryptamine receptors. Br J Pharmacol Chemother 12:323–328

Gozlan H, El Mestikawy S, Pichat L, Glowinsky J, Hamon M (1983) Identification of presynaptic serotonin autoreceptors using a new ligand: [^{3}H]-PAT. Nature 305:140–142

Heuring RE, Peroutka SJ (1987) Characterization of a novel [^{3}H]-5-hydroxytryptamine binding site subtype in bovine brain membranes. J Neurosci 7:894–903

Hoyer D, Engel G, Kalkman HO (1985) Characterization of the 5-HT_{1B} recognition in rat brain: binding studies with (–)-[^{125}I]-iodocyanopindolol. Eur J Pharmacol 118:1–12

Hoyer D, Neijt HC (1987) Identification of serotonin 5-HT_3 recognition sites by radioligand binding in NG 108-15 neuroblastoma-glioma cells. Eur J Pharmacol 143:291–292

Hoyer D, Neijt HC (1988) Identification of 5-HT_3 recognition sites in membranes of NIE-115 neuroblastoma cells by radioligand binding. Mol Pharmacol 33:303–309

Hoyer D, Schoeffter P (1988) 5-HT_{1D} receptor-mediated inhibition of forskolin stimulated adenylate cyclase activity in calf substantia nigra. Eur J Pharmacol 147:145–147

Julius D, MacDermott AB, Axel R, Jessel TM (1988) Molecular characterization of a functional cDNA encoding the serotonin 1C receptor. Science 241:558–564

Kendall DA, Nahorski SR (1985) 5-hydroxytryptamine-stimulated inositol phospholipid hydrolysis in rat cerebral cortex slices: pharmacological characterization and effects of antidepressents. J Pharmacol Exp Ther 233:473–479

Kilpatrick GJ, Jones BJ, Tyers MB (1987) Identification and distribution of 5-HT_3 receptors in rat brain using radioligand binding. Nature (Lond) 330:746–748

Leysen JE, Niemegeers CJE, Van Nueten JM, Laduron PM (1982) [^{3}H]-ketanserin (R41468), a selective [^{3}H]-ligand for serotonin$_2$ receptor binding sites. Mol Pharmacol 21:301–314

Lubbert H, Hoffmann BJ, Snutch P, Van Dyke T, Levine JA, Hartig PR, Lester HA, Davidson N (1987) cDNA cloning of a serotonin 5-HT_{1C} receptor by electrophysiological assays of mRNA injected Xenopus oocytes. Proc Natl Acad Sci [USA] 84:4332–4336

Markstein R, Hoyer D, Engel G (1986) 5-HT_{1A} receptors mediate stimulation of adenylate cyclase in rat hippocampus. Naunyn-Schmiedeberg's Arch Pharmacol 333:335–341

Mawe GM, Branchek TA, Gerhon MD (1986) Peripheral neural serotonin receptors: Identification and characterization with specific antagonists. Proc Natl Acad Sci [USA] 83:9799–9803

Neijt HC, Karpf A, Schoefter P, Engel G, Hoyer D (1988) Characterisation of 5-HT_3 recognition sites in membranes of NG 108-15 neuroblastoma-glioma cells with [^{3}H]-ICS 205930. Naunyn-Schmiedeberg's Arch Pharmacol 337:493–499

Pazos A, Hoyer D, Palacios JM (1984) The binding of serotoninergic ligands to the porcine choroid plexus: Characterization of a new type of serotonin recognition site. Eur J Pharmacol 106:539–546

Pedigo NW, Yamamura HI, Nelson DL (1981) Discrimination of multiple [^{3}H]-5-hydroxytryptamine-binding sites by the neuroleptic spiperone in rat brain. J Neurochem 36:220–226

Peroutka SJ (1988) 5-hydroxytryptamine receptor subtypes: molecular, biochemical and physiological characterization. TINS 11:496–500

Peroutka SJ, Hamik A (1988) [^{3}H]-quipazine labels 5-HT_3 recognition sites in rat cortical membranes. Eur J Pharmacol 148:297–299

Pfeuffer-Friederich I, Kilbinder H (1984) Facilitation and inhibition by 5-hydroxytryptamine and R51619 of acetylcholine release from guinea pig myenteric neurons. In: Roman C (eds) Gastrointestinal Motility. MTP Press Ltd, pp 527–534

Pritchett DB, Bach AWJ, Wozny M, Taleb O, Dal Toso R, Shih JC, Seeburg PH (1988) Structure and functional expression of cloned rat serotonin 5-HT_2 receptor. EMBO J 7:4135–4140

Richardson BP, Engel G, Donatsch P, Stadler PA (1985) Identification of serotonin M-receptor subtypes and their specific blockade by a new class of drugs. Nature 316:126–131

Richardson BP, Buchheit KH (1988) The pharmacology, distribution and function of 5-HT_3 receptors. In: Osborne NN, Hamon M (eds) Neuronal serotonin, vol 17. John Wiley and Sons Ltd, Chichester, pp 465–506

Sanger GJ (1987) Increased gut cholinergic activity and antagonism of 5-hydroxytryptamine M-receptors by BRL24924: potential clinical importance of BRL24924. Br J Pharmacol 91:77–87

Sanger GJ, Nelson DR (1989) Selective and functional 5-hydroxytryptamine$_3$ receptor antagonism by BRL43694 (granisetron). Eur J Pharmacol 159:113–124

Sanger GJ, Wardle KA (1989) 5-hydroxytryptamine and cholinergic function in the gastrointestinal tract. In: Paoletti R, Vanhoutte PM (eds) Serotonin: from cell biology, pharmacology and therapeutics. Kluwer Academic Publishers, Dordrecht. (in press)

Shoeffter P, Waeber C, Palacios JM, Hoyer D (1988) The 5-hydroxytryptamine 5-HT_{1D} receptor subtype is negatively coupled to adenylate cyclase in calf substantia nigra. Naunyn-Schmiedeberg's Arch Pharmacol 337:602–608

Scholtysik G (1987) Evidence for inhibition by ICS 205930 and stimulation by BRL34915 of K^+ conductance in cardiac muscle. Naunyn-Schmiedeberg's Arch Pharmacol 335:692–696

Schuurkes JAJ, Van Nueten JM, Van Deale PGH, Reyntjens AJ, Janssen PAJ (1985) Motor stimulating properties of cisapride on isolated gastrointestinal preparations on the guinea pig. J Pharmacol Exp Ther 234:775–783

Schuurkes JAJ, Megers AAHP, Niemegeers CJE, Leysen JE, Van Nueten JM (1987) A comparative study of the cholinergic vs the anti-dopaminergic properties of benzamides with gastrointestinal prokinetic activity. In: Szurzewski J (ed) Cellular physiology and clinical studies of gastrointestinal smooth muscle. Elsevier, Amsterdam, North Holland, pp 231–247

Shenker A, Maayani S, Weinstein H, Green JP (1987) Pharmacological characterization of two 5-hydroxytryptamine receptors coupled to adenylate cyclase in guinea pig hippocampal membranes. Mol Pharmacol 31:357–367

Van Heuven-Nolsen D (1988) 5-HT receptor subtype specific drugs and the cardiovascular system. TIPS 9:423–425

Waeber C, Schoefter P, Palacios JM, Hoyer D (1988a) Molecular pharmacology of 5-HT_{1D} recognition sites: Radioligand binding studies in human, pig and calf brain membranes. Naunyn-Schmiedeberg's Arch Pharmacol 337:595–601

Waeber C, Dielt MM, Hoyer D, Probst A, Palacios JM (1988b) Visualization of a novel serotonin site 5-HT_{1D} in human brain by autoradiography. Neurosci Lett 88:11–16

Waeber C, Dixon K, Hoyer D, Palacios JM (1988c) Localisation by autoradiography of neuronal 5-HT_3 receptors in mouse CNS. Eur J Pharmacol 151:351–352

Watling K, Aspley S, Swain CJ, Saunders J (1988a) [^{3}H]-Quaternised ICS205930 labels 5-HT_3 receptor binding sites in rat brain. Eur J Pharmacol 149:397–398

Watling KJ (1988b) Radioligand binding studies identify 5-HT_3 recognition sites in neuroblastoma cell lines and mammalian CNS. TIPS 9:227–229

Weiss S, Sebben M, Adolfo-Garcia-Sainz J, Bockaert J (1985) D2-dopamine receptor-mediated inhibition of cyclic AMP formation in striatal neurons in primary culture. Mol Pharmacol 27:595–599

Weiss S, Pin JP, Sebben M, Kemp DE, Sladeczek F, Gabrion J, Bockaert J (1986) Synaptogenesis of cultured striatal neurons in serum-free medium: A morphological and biochemical study. Proc Natl Acad Sci [USA] 83:2238–2242

Weiss S, Sebben M, Kemp D, Bockaert J (1986) Serotonin 5-HT_1 receptors mediate inhibition of cyclic AMP production in neurons. Eur J Pharmacol 120:227–230

Received March 28, 1989/Accepted June 19, 1989

Editorial footnote: In this paper, the term 5-HT_4 receptor is used following the precedent set in previous publications on this subject (Dumuis et al. 1988b, 1989). It is recognised, however, that this nomenclature is not established and currently under debate by the Nomenclature Committee of the Serotonin Club