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5-Hydroxytryptamine$_4$ receptors mediate relaxation of the rat oesophageal tunica muscularis mucosae

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Summary. The present study was designed to characterize an "atypical" 5-hydroxytryptamine (5-HT) receptor mediating relaxation of the rat oesophageal tunica muscularis mucosae. All experiments were performed under equilibrium conditions, using pargyline to inhibit the oxidative deamination of indoleamines, and cocaine and corticosterone to inhibit neuronal and extraneuronal uptake. Under these conditions 5-HT (0.3–1000 nmol/l) produced a concentration-dependent relaxation of carbachol-induced tension. The concentration-effect curve to 5-HT was unaffected by potent antagonists for 5-HT$_1$, 5-HT$_2$, 5-HT$_3$ and so called 5-HT$_{1P}$ receptors (metergoline, methysergide, ketanserin, ondansetron, *N*-acetyl-5-hydroxytryptophyl-5-hydroxytryptophan amide), but was antagonized competitively by ICS 205-930 (pA_2 = 6.7). Responses to 5-HT were mimicked by other indoleamines and substituted benzamides with the following order of potency: 5-HT ≥ 5-methoxytryptamine > cisapride = α-methyl-5-HT = (*S*)-zacopride = renzapride > (*RS*)-zacopride > 5-carboxamidotryptamine = metoclopramide = (*R*)-zacopride > tryptamine > 2-methyl-5-HT. ICS 205-930 afforded similar pA_2 values (6.0–6.7) against each agonist, indicating a common site of action. Concentration-effect curves to 5-HT were not affected by tetrodotoxin or indomethacin, sugesting that 5-HT-induced relaxation of the tunica muscularis mucosae was mediated via a postjunctional receptor, independent of endogenous prostanoids. The pharmacological profile of the 5-HT receptor in the rat oesophageal tunica muscularis mucosae correlates well with the 5-HT$_4$ receptor characterized recently in both the CNS and gastro-intestinal tract.

Key words: 5-HT$_4$ – Oesophagus – Rat – ICS 205-930 – Benzamides

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Introduction

The novel 5-hydroxytryptamine$_4$ (5-HT$_4$) receptor, named by Dumuis et al. (1988a) and located in mouse embryonic colliculi neurones and guinea-pig hippocampus, is characterized by a high sensitivity to both 5-HT and 5-methoxytryptamine (5-MeO-T) as well as by agonistic activity of certain substituted benzamides, including renzapride, cisapride and metoclopramide (Dumuis et al. 1989a, b; Bockaert et al. 1990a). Additionally, the site demonstrates a low affinity (pK_i estimates in the range 6.0–6.3) for the potent 5-HT$_3$ receptor antagonist ICS 205-930, a finding which initially led Bockaert's group (Dumuis et al. 1988b) to suggest that they might have discovered an embryonic form of the 5-HT$_3$ receptor.

Several other investigators have since reported the existence of peripheral receptor sites for 5-HT which display a similar pharmacological profile to the 5-HT$_4$ receptor in the CNS. These include neuronally located receptors in guinea-pig ileum (Craig and Clarke 1989; Clarke et al. 1989; Craig and Clarke 1990; Eglen et al. 1990) and colon (Elswood et al. 1990) and receptors influencing cardiac contractility in both pig (Villalón et al. 1990; Kaumann 1990) and man (Kaumann et al. 1990a, b). In this regard an "atypical" 5-HT receptor, which exhibits certain pharmacological characteristics of a 5-HT$_4$ receptor, has been identified in rat oesophagus (Triggle et al. 1988; Reeves et al. 1989). In preparations of oesophageal tunica muscularis mucosae, 5-HT causes relaxation via a non-neuronal mechanism which is resistant to 5-HT$_1$ and 5-HT$_2$ antagonists. However, questions remain as to the exact nature of the receptor involved. Thus, Triggle et al. (1988) reported that the 5-HT$_3$ antagonists ICS 205-930, MDL 72222 and granisetron inhibited the relaxant responses to 5-HT in rat oesophagus, whereas Reeves et al. (1989) found MDL 72222 and granisetron to be inactive. Both groups reported unsurmountable antagonism toward 5-HT with substituted benzamides. In addition, Reeves et al. (1989) found

5-MeO-T to be an inordinately weak agonist, being over 100 times less potent than 5-HT.

The present study was designed to characterize more fully the receptor mediating 5-HT-induced relaxation in rat oesophagus. Care was taken to work under equilibrium conditions so as to obtain unequivocal agonist potency ratios and pA_2 values. A preliminary acount of this work was presented at the Second IUPHAR Satellite Meeting on Serotonin, Basel, Switzerland, July 1990.

Materials and methods

Male Sprague-Dawley rats (200 – 300 g) were killed by asphyxiation with CO_2, and a 2-cm segment of intra-thoracic oesophagus, proximal to the diaphragm, was excised and placed in Tyrode's solution of the following composition (mmol/l): NaCl (136), KCl (2.7), $MgCl_2 \cdot 6\,H_2O$ (1.0), $NaH_2PO_4 \cdot H_2O$ (0.4), glucose (5.6), $NaHCO_3$ (11.9) and $CaCl_2$ (1.8), pH 7.4. The external muscularis propria, containing the outer longitudinal and circular muscle layers of the oesophagus, was carefully removed in order to isolate the inner smooth muscle tube of the tunica muscularis mucosae. This was suspended in a 10-ml tissue bath containing Tyrode's solution at 37°C and aerated continuously with 95% O_2/5% CO_2. Tissues were placed under 2.5-mN tension and were left to equilibrate with Tyrode's solution for 60 min (washing every 15 min) prior to starting the experiment. Responses were recorded isometrically using a Hugo Sachs Elektronik (Biegestab K30) transducer coupled to a Graphtec (linearcorder WR3310) four-channel chart recorder.

Experimental protocol and concentration-effect curves. Several experiments were undertaken to account for tissue factors which may influence agonist concentration at the receptor of interest. The findings are given in "Results" and led to the adoption of the following experimental conditions. Rat oesophagi were incubated with pargyline (100 μmol/l) for 30 min to inactivate monoamine oxidases, and both cocaine (30 μmol/l) and corticosterone (30 μmol/l) were included in the Tyrode's solution to inhibit the uptake of 5-HT. In addition, all experiments were conducted in the presence of methysergide (1 μmol/l) to block 5-HT_1 and 5-HT_2 receptors.

Concentration-effect curves were obtained after contracting the rat oesophagus with carbachol (3 μmol/l; a concentration evoking 80% of the maximum contraction). Responses to the cumulative addition of agonists are expressed as percentage relaxation of the carbachol-induced tone. Using this procedure, a complete concentration-effect curve to 5-HT was obtained with a maximum relaxation of approximately 80% of the initial contraction. This allowed for the expression of agonists with a greater intrinsic activity than 5-HT while giving a large concentration-response signal to 5-HT itself.

All agonist concentration-effect curves were fitted using a non-linear iterative fitting program (Kaleidagraph, Synergy Software, PCS Inc., Reading, Pa.) according to the following relationship (Parker and Waud 1971):

$$E = M[A]^p/([A]^p + [K]^p).$$

This relationship describes a curve with maximum response M, an EC_{50} equal to K and a slope determined by the power p. [A] represents agonist concentration and E is response.

Agonist potency. The ability of agonists to relax rat oesophagus is expressed both in absolute terms, as EC_{50} values (relative to their individual maxima), and in terms of their relative potency versus 5-HT. Potency relative to 5-HT was calculated from experiments in which two concentration-effect curves were constructed in the same preparation: the first to 5-HT itself and the second to either 5-HT (in time control experiments) or to a test agonist. The relative potency of agonists is expressed as equipotent concentration ratios (ECR) measured at the 40% inhibition point (IC_{40}) of the carbachol-induced contraction, a point which is approximately equivalent to the EC_{50} of the concentration-effect curve to 5-HT. ECR values were calculated as follows:

$$ECR = IC_{40}\text{ test agonist}/IC_{40}\text{ 5-HT}.$$

Antagonist potency. Values of pA_2 for ICS 205-930 versus 5-HT were determined by the method of Arunlakshana and Schild (1959) and computed using Statview 512+ (Brain Power Inc., Calabasas, Calif.). Two concentration-effect curves were obtained in each preparation, the first in the absence, and the second in the presence of ICS 205-930. Rat oesophagi were incubated with ICS 205-930 for 90 min prior to construction of the second concentration-effect curve. Concentration ratios were measured relative to the EC_{50} of the first concentration-effect curve.

Values of pA_2 for ICS 205-930 versus all other agonists were determined by the method of Furchgott (1972) using a single concentration of antagonist. The method assumes a competitive interaction and is calculated as follows:

$$pA_2 = \log[\text{antagonist concentration (mol/l)}/(\text{agonist concentration ratio} - 1)].$$

Compounds used. The following drugs were purchased from the suppliers indicated: 5-hydroxytryptamine hydrochloride, reserpine, corticosterone, carbamylcholine chloride, tryptamine hydrochloride, tetrodotoxin, indomethacin, substance P and cocaine hydrochloride (Sigma Chemical Co., St. Louis, Mo., USA); α-methyl-5-hydroxytryptamine maleate, 2-methyl-5-hydroxytryptamine maleate and metoclopramide (Research Biochemicals Inc., Natick, Mass., USA); 5-methoxytryptamine hydrochloride (Aldrich Chemical Co., St. Louis, Mo., USA). The following compounds were obtained as gifts: metergoline (Farmitalia, Milano, Italy); renzapride (Dr. G. Sanger, Beecham Pharmaceuticals, Harlow, Essex, UK); *N*-acetyl-5-hydroxytryptophyl-5-hydroxytryptophan amide (Dr. M. D. Gershon, Columbia University College of Physicians and Surgeons, New York, NY, USA, and Dr. H. Tamir, New York Psychiatric Institute, New York, NY, USA); 5-carboxamidotryptamine maleate and ondansetron (Dr. P. P. A. Humphrey, Glaxo Group Research, Ware, Herts., UK); cisapride and ketanserin (Dr. J. Leysen, Jannsen Pharmaceutica, Beerse, Belgium); ICS 205-930 ([3*a*-tropanyl]-1*H*-indole-3-carboxylic acid ester), (Drs. J. Fozard and D. Hoyer, Sandoz, Basel, Switzerland); methysergide and the isomers of zacopride (Dr. R. Clark, Syntex Research, Palo Alto, Calif., USA). All stock solutions of drugs were made up in distilled water with the following exceptions: corticosterone (dimethylsulphoxide:water, 50:50, v:v); cisapride (methanol); *N*-acetyl-5-HTP-DP (propylene glycol:water, 10:90, v:v) and indomethacin (10% $NaHCO_3$).

Results

Preliminary experiments

The cumulative addition of 5-HT (0.001 – 1 μmol/l) caused a concentration-dependent relaxation of the rat oesophagus pre-contracted with carbachol (3 μM). Concentration-effect curves to 5-HT were not influenced by methysergide (1 μmol/l) or several other 5-HT receptor antagonists: metergoline (3.0 μmol/l), ketanserin (3 μmol/l), ondansetron (5 μmol/l) and *N*-acetyl-5-HTP-DP (1 μmol/l), indicating little or no involvement of 5-HT_1, 5-HT_2, 5-HT_3 or 5-HT_{1P} receptors (Humphrey 1984; Hoyer 1985; Mawe et al. 1986; Butler et al. 1988). Despite the ineffectiveness of the antagonists, methysergide (1 μmol/l) was added routinely to Tyrode's

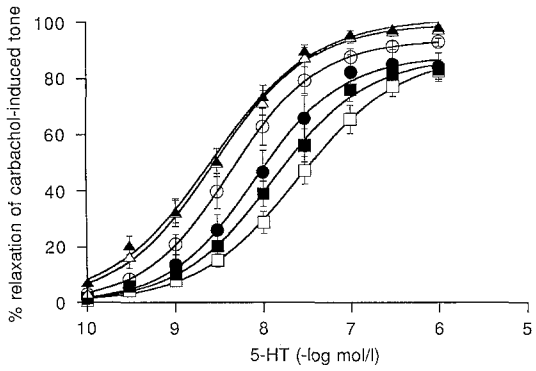

Fig. 1. Cumulative concentration-effect curves to 5-HT in the absence (□) and presence of 3.0 (■), 10.0 (●), 30.0 (○), 100.0 (△) and 300 (▲) μmol/l cocaine in rat oesophageal tunica muscularis mucosae. Each *point* represents the arithmetic mean ± SE of the ratio from 6 experiments

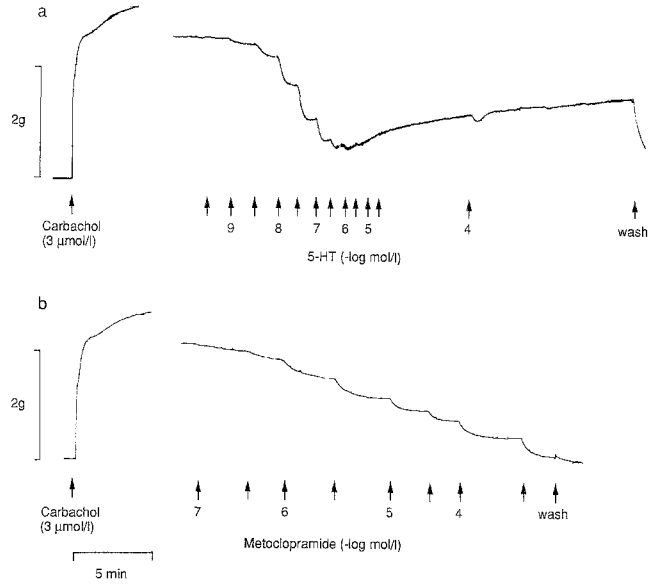

Fig. 2a, b. Cumulative concentration-effect curves to **a** 5-HT (0.3 nmol/l – 100 μmol/l) and **b** metoclopramide (0.1 – 300 μmol/l) in rat oesophageal tunica muscularis mucosae precontracted with carbachol (3 μmol/l)

solution to prevent possible 5-HT_2 receptor-mediated contraction of the oesophagus (Akbarali et al. 1987). The relaxant response to 5-HT was resistant to inhibition by tetrodotoxin and indomethacin, indicating that it is non-neuronal in origin and is independent of the release of prostanoids (Kao 1966; Davis 1976).

Figure 1 shows that responses to 5-HT were potentiated by increasing concentrations of cocaine (3 – 300 μmol/l) with a maximum shift in the concentration-effect curve of eight- to tenfold. At concentrations of 30 μmol/l and below, cocaine appeared selective for 5-HT and did not alter the concentration-effect curves to either papaverine, isoprenaline or carbachol (data not shown). As the higher concentrations of cocaine caused a two- to threefold rightward shift in carbachol concentration-effect curves the lower, 30 μmol/l concentration of cocaine was selected for routine use. Corticosterone (30 μmol/l), in the presence of cocaine (30 μmol/l), produced a small additional (threefold) leftward shift in the concentration-effect curve to 5-HT and was included routinely in Tyrode's solution. In contrast, the concentration-effect curve to 5-HT was unaffected by pretreatment of the rat oesophagus with the irreversible monoamine oxidase inhibitor pargyline (100 μmol/l for 30 min, followed by a 30-min washout). However, in view of the propensity of the more lipophilic analogues of 5-HT to deamination by monoamine oxidase, pargyline was used routinely in all subsequent experiments. Finally, concentration-effect curves for neither 5-HT nor tryptamine were affected by pretreatment of rats with reserpine (5 mg/kg i.p. for 18 h before use), suggesting that little or no part of the response to these agonists was mediated indirectly via the release or endogenous monoamines. As a result, reserpine pretreatment was not used routinely.

Tryptamine analogues

In experiments carried out in pargyline-treated preparations in the presence of cocaine (30 μmol/l), corticosterone (30 μmol/l) and methysergide (1 μmol/l), the cumulative addition of 5-HT (0.1 – 100 nmol/l) to the

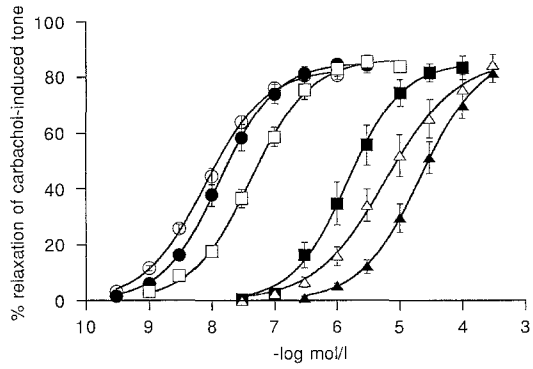

Fig. 3. Cumulative concentration-effect curves to 5-HT (○), 5-MeO-T (●), α-methyl-5-HT (□), 5-CT (■), tryptamine (△), and 2-methyl-5-HT (▲) in rat oesophageal tunic amuscularis mucosae. Each *point* represents the arithmetic mean ± SE of the ratio (see Table 1 for number of experiments)

carbachol-contracted rat oesophagus caused a concentration-dependent relaxation with a mean pEC_{50} (95% CL) of 8.2 (8.1 – 8.3) ($n = 39$). At low concentrations, responses to 5-HT were slow in onset, whereas at higher concentrations a steady state was reached more quickly (Fig. 2a). The maximum relaxation with 5-HT occurred at 1 μmol/l. The further addition of 5-HT failed to produce responses at concentrations up to 30 μmol/l. In the continued presence of 5-HT (30 μmol/l) the preparations exhibited desensitization, which was characterized by a slow, but incomplete return of the carbachol-induced tone. Subsequent addition of a high concentration of 5-HT (100 μmol/l) produced a transient relaxation.

All tryptamine analogues acted as full agonists on the rat oesophagus, and with the possible exception of tryptamine itself, produced parallel concentration-effect curves to 5-HT (Fig. 3). 5-MeO-T was approximately

Table 1. Agonist potency in rat esophageal tunica muscularis mucosae

Agonist	pEC_{50} (95% CL)	Intrinsic activity	n	ECR[a]	n
5-HT	8.2 (8.1–8.3)	1.00	39	1	39
5-MeO-T	7.9 (7.8–8.0)	1.00	8	2	3
α-Methyl-5-HT	6.9 (6.7–7.0)	1.00	12	10	4
Cisapride	7.6 (7.4–7.7)	0.84	9	12	6
(*S*)-Zacopride	7.2 (7.1–7.2)	0.90	20	12	3
Renzapride	7.2 (6.9–7.5)	0.80	7	21	3
(*RS*)-Zacopride	6.6 (6.3–7.0)	0.91	7	86	3
5-CT	5.8 (5.5–6.1)	1.00	6	95	4
Metoclopramide	6.1 (5.9–6.4)	0.80	6	128	3
(*R*)-Zacopride	6.3 (6.1–6.5)	0.90	12	130	3
Tryptamine	5.1 (4.7–5.6)	1.00	8	906	4
2-Methyl-5-HT	4.7 (4.4–4.9)	1.00	6	1548	3

Each value represents the arithmetic mean with 95% confidence limits in parentheses. [a] ECR values = IC_{40} test agonist ÷ IC_{40} 5-HT, and were limited to experiments in which concentration-effect curves for both agonists were obtained in the same preparation (for further details see "Materials and methods")

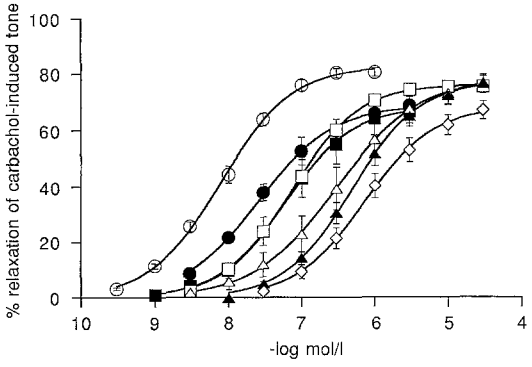

Fig. 4. Cumulative concentration-effect curves to 5-HT (○), cisapride (●), (*S*)-zacopride (□), renzapride (■), (*RS*)-zacopride (△), (*R*)-zacopride (▲) and metoclopramide (◇) in rat oesophageal tunica muscularis mucosae. Each *point* represents the arithmetic mean ± SE of the ratio (see Table 1 for number of experiments)

equipotent with 5-HT, whereas 5-CT (a 5-HT_1 receptor agonist; Bradley et al. 1986), α-methyl-5-HT (a 5-HT_2 receptor agonist; Richardson et al. 1985) and 2-methyl-5-HT (a 5-HT_3 receptor agonist; Humphrey 1984; Bradley et al. 1986) were approximately 10, 90 and 1500 times less potent than 5-HT respectively (Table 1).

2-methoxy-4-amino-5-chloro-substituted benzamides

The 2-methoxy-4-amino-5-chloro-substituted benzamides [cisapride, renzapride, (*S*)-zacopride, (*R*)-zacopride, (*RS*)-zacopride and metoclopramide] were less potent agonists than 5-HT in relaxing the oesophagus (Table 1; Fig. 4) and were noticeably slower to attain steady state than 5-HT (compare Fig. 2a with Fig. 2b). All benzamides exhibited a second inhibitory phase to their concentration-effect curves (Figs. 2b, 5). With cisapride this second phase was evident at concentrations

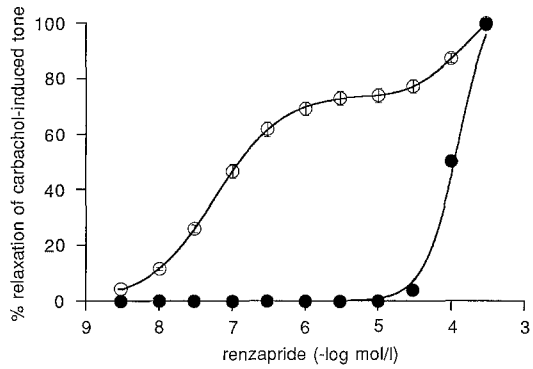

Fig. 5. Cumulative concentration-effect curves to renzapride in the absence (○) and presence (●) of 5-MeO-T (100 μmol/l) in rat oesophageal tunica muscularis mucosae. Each *point* represents the arithmetic mean ± SE of the ratio for 6 experiments

of 10 μmol/l and above; however, with the remaining benzamides it was observed only when concentrations exceeded 30 μmol/l.

Figure 4 shows fitted data points for the first phase of the concentration-effect curves to the substituted benzamides. It can be seen that all benzamides acted as partial agonists, evoking approximately 80–90% of the maximum response to 5-HT (see also Table 1). The second, low-potency phase of the benzamide concentration-effect curves appeared unrelated to the activation of 5-HT receptors. Thus, desensitization of the 5-HT receptor with 5-MeO-T (10 μmol/l for 60 min; Craig et al. 1990) abolished responses to 5-HT (up to 30 μmol/l) and inhibited the first phase of the concentration-effect curve to the benzamides, whereas the second phase persisted. This latter point is illustrated for renzapride in Fig. 5. In other experiments, in which 5-HT receptors were desensitized with 5-MeO-T (10 μmol/l), a high concentration of (*R*)-zacopride (30 μmol/l) was found to inhibit responses induced by carbachol but not those induced by substance P (data not shown).

ICS 205-930

ICS 205-930 (0.3–3.0 μmol/l) caused a parallel rightward displacement of the concentration-effect curves to 5-HT (Fig. 6a). The slope of the Schild regression (1.06, 95% CL = 0.74–1.4) was not significantly different from unity and yielded an estimated pA_2 of 6.7, with the slope constrained to 1 (Fig. 6b). Estimates of the pA_2 value for ICS 205-930 against the remaining indoleamines and the substituted benzamides ranged from 6.0 to 6.7 (Table 2).

A higher concentration of ICS 205-930 (10 μmol/l) produced a smaller dextral displacement of the concentration-effect curves to 5-HT than predicted from the Schild analysis, suggesting the presence of a secondary effect of ICS 205-930 (Fig. 6a). Indeed, muscarinic cholinoceptor antagonism by ICS 205-930, with a pA_2 estimate of 5.4 (95% CL = 5.2–5.6), was determined in other experiments. This pharmacological property of ICS 205-930 may give rise to an underestimate of its affinity at 10 μmol/l owing to functional synergism with the in-

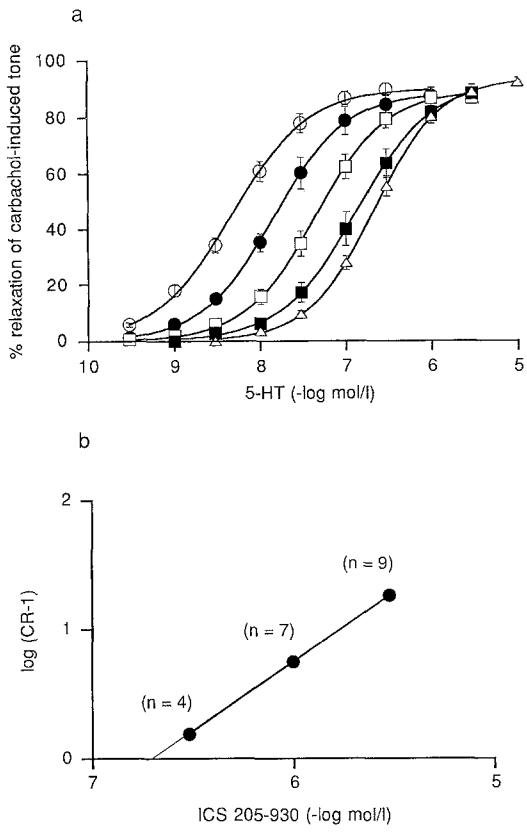

Fig. 6. **a** Cumulative concentration-effect curves to 5-HT in the absence (○) and presence of 0.3 (●), 1.0 (□), 3.0 (■) and 10.0 (△) μmol/l ICS 205-930 in rat oesophageal tunica muscularis mucosae. **b** Schild regression analysis derived from agonist concentration ratios at 0.3, 1.0 and 3.0 μmol/l ICS 205-930. Each *point* represents the arithmetic mean ± SE of the ratio for 4–7 experiments (see "Results" for further details)

Table 2. pA_2 estimates for ICS 205-930 in rat esophageal tunica muscularis mucosae

Agonist	pA_2 (95% CL)[a]	*n*
5-HT	6.7[b]	6
5-MeO-T	6.5 (6.0–6.8)	6
Cisapride	6.5 (6.1–6.9)	3
(*S*)-Zacopride	6.5 (5.6–7.4)	3
α-Methyl-5-HT	6.7 (6.2–7.1)	4
Renzapride	6.2 (5.9–6.4)	3
5-CT	6.0 (4.7–7.3)	3
Metoclopramide	6.1 (5.8–6.3)	4
(*R*)-Zacopride	6.4 (6.1–6.7)	3
2-Methyl-5-HT	6.1 (5.4–6.8)	3
Carbachol	5.4[b] (5.2–5.6)	6
Isoprenaline	< 5.0	6

[a] Single point analysis with 3 μmol/l ICS 205-930
[b] Schild regression analysis with slope constrained to 1

hibitory responses to 5-HT in the carbachol-contracted oesophagus. However, the same concentration of ICS 205-930 failed to alter the inhibitory potency of isoprenaline significantly. Thus, the notion of functional synergism seems unlikely, and the reason for deviation from competitive kinetics by the highest concentration of ICS 205-930 remains unresolved.

Discussion

The aim of the present study was to characterize pharmacologically the atypical 5-HT receptor in the tunica muscularis mucosae of the rat oesophagus. To achieve this aim it was necessary to isolate the receptor of interest and to conduct experiments under equilibrium conditions (Furchgott 1972). Although 5-HT is subject to neuronal and extraneuronal uptake, both mechanisms may be inhibited by cocaine (Verbeuren et al. 1983; Fukuda et al. 1986). Cocaine caused a concentration-dependent leftward shift in the concentration-effect curves to 5-HT, consistent with the presence of a cocaine-sensitive uptake system for 5-HT (Thoa et al. 1969; Verbeuren et al. 1983; Paiva et al. 1984; Fukuda et al. 1986) in rat oesophagus. At a concentration of 30 μmol/l, cocaine caused about a sevenfold shift in the concentration-effect curve to 5-HT and appeared to act selectively, as the concentration-effect curves to both isoprenaline and papaverine were unaffected. However, higher concentrations of cocaine antagonized responses to carbachol, possibly as a result of its local anaesthetic action (Fozard et al. 1979). Kaumann et al. (1990a, b) have reported that cocaine caused a five- to sixfold increase in the potency of 5-HT at the 5-HT_4 receptor in the human atrial appendage, whereas responses to renzapride were unaffected. This suggests that the potentiating action of cocaine is not mediated allosterically via the 5-HT_4 receptor and that uptake inhibition is the most likely mechanism.

Both 5-HT (Humphrey et al. 1983) and tryptamine (Takaki et al. 1985) can release endogenous indoleamines and catecholamines, which may result in erroneous estimates of agonist potency. However, in the present study depletion of endogenous monoamines with reserpine did not influence the concentration-effect curves to 5-HT or tryptamine, suggesting that such a mechanism is not operative in rat oesophagus under the conditions employed. Similarly, an indirectly mediated response via prostaglandin synthesis and release (Davis 1976) seems unlikely, as concentration-effect curves to 5-HT were unaffected by the cyclo-oxygenase inhibitor indomethacin. In view of the negative results with both reserpine and indomethacin, their routine use was considered unnecessary. In contrast, the use of the irreversible monoamine oxidase inhibitor pargyline was continued, despite its ineffectiveness against 5-HT, as the possibility exists that the more lipophilic indoleamines may diffuse across cell membranes to be deaminated intracellularly, thereby creating a metabolic sink.

Although evidence exists for functional neurones in the tunica muscularis mucosae of the rat oesophagus (Akbarali et al. 1986; Bieger and Triggle 1985), responses to exogenously applied 5-HT in the present study were not inhibited by tetrodotoxin. Thus the 5-HT_4 receptor in rat oesophagus appears to be located post-junctionally. This is certainly not the case in the guinea-pig ileum where the 5-HT_4 receptor is located neuronally and mediates

the release of acetylcholine (Craig and Clarke 1990) or possibly substance P (Buchheit et al. 1985) or both. It is not clear whether the 5-HT_4 receptor in human (Kaumann et al. 1990a, b) or pig (Villalón et al. 1990; Kaumann 1990) cardiac tissue is neuronally located, but in guinea-pig right atria it is inhibited by capsaicin, suggesting a neuronal location (R. M. Eglen, unpublished observations).

Preliminary studies with methysergide and other 5-HT antagonists failed to provide evidence for 5-HT_1, 5-HT_2 or 5-HT_3 receptors in rat oesophagus. Other investigators have reported the exposure of a weak ketanserin-sensitive contraction to 5-HT in rat oesophagus following storage at 4° C (Akbarali et al. 1987). Although no such contractions were observed in the present study, which utilized fresh, non-cooled tissues, methysergide was included routinely in Tyrode's solution to counter any possible expression of 5-HT_2 receptors. In addition, it is pertinent to note that the concentration of cocaine used in the present study is approximately 30-fold higher than its equilibrium dissociation constant for 5-HT_3 receptors (Fozard et al. 1979), thus providing a substantial 5-HT_3 blocking activity should such receptors also become revealed. In effect, therefore, any possible interaction of agonists at 5-HT_1, 5-HT_2 or 5-HT_3 receptors was inhibited by the combination of methysergide and cocaine. Furthermore, no evidence was obtained for the presence of the so called 5-HT_{1P} receptor in the rat oesophagus. Such receptors are inhibited by *N*-acetyl-5-HTP-DP and are resistant to inhibition by ICS 205-930 (Mawe et al. 1986). Thus, from an antagonist standpoint, the 5-HT_4 receptor is defined largely by exclusion. This is not a desirable situation, and selective competitive antagonists of high affinity are awaited.

After taking steps to ensure equilibrium conditions and to pharmacologically isolate the atypical 5-HT receptor, an attempt was made to obtain an agonist "fingerprint" for the site. 5-MeO-T was found to be approximately equipotent with 5-HT, whereas α-methyl-5-HT, 5-CT and 2-methyl-5-HT were approximately, 10, 100 and over 1000 times less potent than 5-HT respectively. The potency order for these indoleamines does not reflect those established previously at 5-HT_1, 5-HT_2 and 5-HT_3 receptor subtypes (Humphrey 1984; Bradley et al. 1986; Leff and Martin 1988) and, coupled with results from antagonist studies, supports the notion that the rat oesophagus expresses a novel 5-HT receptor.

The results obtained with the indole agonists reflect those reported at the 5-HT_4 receptor in both the CNS and the periphery (Dumuis et al. 1988a; Craig and Clarke 1990; Eglen et al. 1990). However, there are some clear differences when comparisons are drawn with the results of Reeves et al. (1989) in rat oesophagus, and those of Hill et al. (1990) in guinea-pig ileum. In these studies the potency of 5-MeO-T was found to be considerably lower than 5-HT, and in the rat oesophagus 5-CT was found to be more potent than 5-MeO-T. The reason for these discrepancies is unknown. In the present study and in those of others (Dumuis et al. 1988a; Craig and Clarke 1990; Eglen et al. 1990), 5-MeO-T exhibited a potency of approximate equivalency to 5-HT and was always 10–40 times as potent than 5-CT.

Agonism by certain substituted benzamides represents a key pharmacological characteristic which defines and distinguishes the 5-HT_4 receptor from all other subtypes of 5-HT receptor. Although the benzamides also possess affinity for 5-HT_3 receptors, they lack intrinsic efficacy (Schuurkes et al. 1985; Sanger and King 1988). In the present study, cisapride, renzapride and (*S*)-zacopride were only about tenfold less potent than 5-HT at the 5-HT_4 receptor, whereas metoclopramide, (*RS*)-zacopride and (*R*)-zacopride were over 80 times less potent. Furthermore, it is significant that the 5-HT receptor in the rat oesophagus discriminates between (*S*)- and (*R*)-zacopride, with the (*S*)-isomer being approximately tenfold more potent than the (*R*)-form. This finding parallels that made by Eglen et al. (1990) at the 5-HT_4 receptor in guinea-pig ileum. Finally, it is noteworthy that the potency of cisapride relative to 5-HT exhibits tissue-dependent differences. Thus, in guinea-pig ileum cisapride is 120 times less potent than 5-HT (Craig and Clarke 1990), in rat oesophagus (this study) and isolated right atria of the piglet (Kaumann 1990) it is approximately 10 times less potent, whereas it is equipotent with 5-HT in mouse colliculi neurones (Dumuis et al. 1989b) and guinea-pig hippocampal membranes (Bockaert et al. 1990a). Whether this tissue dependence represents differences in the 5-HT_4 receptor or arises through other causes remains to be determined.

In general the agonist potency order of the substituted benzamides correlates well with observations made by several independent investigators (Dumuis et al. 1989b; Craig and Clarke 1990; Eglen et al. 1990; Kaumann 1990). However, both Triggle et al. (1988) and Reeves et al. (1989) found renzapride, cisapride and metoclopramide to act as unsurmountable antagonists toward 5-HT in rat oesophagus. These differences may result from a combination of factors related to their agonist properties, including receptor desensitization and their lower intrinsic efficacy relative to 5-HT. In addition, as discussed below, the substituted benzamides may possess muscarinic cholinoceptor blocking activity.

All substituted benzamides gave rise to biphasic concentration-effect curves. The first, and by far the largest, phase of the curves was abolished by desensitization with 5-MeO-T, indicating that it emanated from 5-HT_4 receptor agonism (Craig et al. 1990). However, the second, low-potency phase was resistant to desensitization with 5-MeO-T. As high concentrations of (*R*)-zacopride antagonized contractile responses to carbachol, but not those to substance P, it is likely that the second phase of the concentration-effect curve to (*R*)-zacopride (and possibly the other benzamides) results from muscarinic cholinoceptor antagonism. This finding emphasizes a potential danger of using high concentrations of the substituted benzamides as probes for the 5-HT_4 receptor. Unlike close analogues of 5-HT, large non-indole structures, such as the benzamides, are liable to possess a broader pharmacological profile encompassing actions which fall outside those at 5-HT receptors. Such non-specificity may account for the recent disclosure of a benzamide binding

site in the CNS at which 5-HT lacks affinity (Bockaert et al. 1990b). Likewise, the same explanation may account for the tissue-dependent differences in the potency of cisapride, as discussed above.

Further evidence for the existence of the 5-HT_4 receptor in the rat oesophagus stems from studies with ICS 205-930. To date, ICS 205-930 is the only known competitive antagonist for the 5-HT_4 receptor, but its use is hindered by low affinity at this site compared with the 5-HT_3 receptor (Craig et al. 1990), as well as by effects unrelated to 5-HT, such as blockade of potassium, sodium and calcium currents (Scholtysik et al. 1988). In addition, as demonstrated in the present study, the affinity of ICS 205-930 for muscarinic cholinoceptors ($pA_2 = 5.4$) lies only approximately tenfold below that for the 5-HT_4 receptor (Dumuis et al. 1989b; Craig and Clarke 1990; Craig et al. 1990; Eglen et al. 1990; Kaumann et al. 1990a, b). Nevertheless, this antagonist, at 0.3, 1.0 and 3.0 μmol/l, exhibited competitive kinetics versus 5-HT, yielding an estimated pA_2 value of 6.7. Furthermore, ICS 205-930 (3 μmol/l) did not influence concentration-effect curves to isoprenaline and demonstrated an agonist-independent affinity within the range reported for 5-HT_4 receptors. A broad range of antagonists, both for 5-HT receptors and other receptor systems, have been examined previously in the rat oesophagus (Bieger and Triggle 1985), guinea-pig ileum (Eglen et al. 1990), human atrial appendage (Kaumann et al. 1990b) and right atria of piglets (Kaumann 1990). However, in all of these studies, competitive antagonism at the 5-HT_4 receptor was observed only with ICS 205-930.

The pharmacological profile of the 5-HT_4 receptor described in the present study reveals a remarkable consensus, with few exceptions, with that reported in the literature. This concordance suggests that the 5-HT_4 receptor is a discrete and highly definable pharmacological entity, even though its pharmacology is biased heavily toward its agonist profile. At present, despite tissue-dependent differences in the potency of cisapride, no compelling *body* of evidence exists to suggest heterogeneity of the 5-HT_4 receptor subtype.

Finally, it is pertinent to note that the 5-HT_4 receptor is by far the most predominant, and perhaps the only, 5-HT receptor type expressed in the rat oesophagus. Thus, the rat oesophagus is devoid of the problems associated with the presence of multiple 5-HT receptor subtypes, which may cause confusion in other preparations e.g. guinea-pig ileum (Craig and Clarke 1989) and guinea-pig hippocampus (Shenker et al. 1987). Also, the post-junctional location of the 5-HT_4 receptor in the oesophagus avoids complications associated with indirect, neuronally mediated responses, as is the case with acetylcholine and perhaps, substance-P release in the gastro-intestinal tract of guinea-pigs. As such, use of the tunica muscularis mucosae of the rat oesophagus is a considerable improvement over the currently available screening assays for 5-HT_4 receptor ligands and should prove useful not only to rationalize the actions of the prokinetic benzamides but also for the development of novel therapeutic agents.

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