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Characterization of 5-HT_3 and 'atypical' 5-HT receptors mediating guinea-pig ileal contractions *in vitro*

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1 Neuronal 5-hydroxytryptamine (5-HT) receptors mediating contraction of guinea-pig ileal segments have been characterized *in vitro* by the use of methysergide to block 5-HT_1-like and 5-HT_2 receptors. Concentration-response curves to 5-HT were biphasic (first phase, defined as those responses occurring between 1 nM and 0.32 μM 5-HT, $-\log EC_{50} = 7.15 \pm 0.08$; second phase, defined as these responses occurring between 0.32 μM and 32 μM 5-HT, $-\log EC_{50} = 5.32 \pm 0.03$) but monophasic to 5-methoxytryptamine ($-\log EC_{50} = 7.0 \pm 0.08$) and 2 methyl 5-HT ($-\log EC_{50} = 5.2 \pm 0.13$). The maximal response of the first phase to 5-HT and the maximal response to 5-methoxytryptamine were $30 \pm 4\%$ and $35 \pm 5\%$ respectively of the maximum response to the second phase of the 5-HT concentration-effect curve (set at 100%). In contrast, the maximal response to 2-methyl-5-HT equalled that obtained with 5-HT (second phase).

2 The responses comprising the second phase of the concentration-effect curve to 5-HT were antagonized by 1 μM ICS 205-930, ondansetron, granisetron, quipazine, N-methyl-quipazine and (**R,S**)-zacopride and the following pK_B values, with 5-HT as the agonist, were obtained at the 5-HT_3 receptor: ICS 205-930 7.61 ± 0.05, ondansetron 6.90 ± 0.04, granisetron 7.90 ± 0.04, (**S**)-zacopride 8.11 ± 0.06, (**R,S**)-zacopride 7.64 ± 0.11, and (**R**)-zacopride 7.27 ± 0.06.

3 Under conditions of 5-HT_1-like, 5-HT_2 and 5-HT_3 receptor blockade, the following rank order of agonism was observed: 5-HT > 5-methoxytryptamine = renzapride > (**S**)-zacopride > (**R,S**)-zacopride > 5-carboxamidotryptamine > BRL 24682 > (**R**)-zacopride > metoclopramide > 2-methyl-5-HT $\gg$ sulpiride. 8-Dihydroxydiphenylaminotetralin (8-OHDPAT), GR 43175, N,N-dipropyl-5-carboxamidotryptamine, ondansetron, ICS 205-930, granisetron, quipazine and N-methyl-quipazine were inactive as agonists and antagonists. Relative to 5-HT, (**R,S**)-zacopride acted as a partial agonist (intrinsic activity, $\alpha = 0.80$; $-\log EC_{50} = 6.3 \pm 0.12$; $-\log K_A = 6.1 \pm 0.03$) as did (**R**)-zacopride ($\alpha = 0.4$, $-\log EC_{50}$ 5.7 ± 0.08, $-\log K_A = 5.5 \pm 0.11$). (**S**)-zacopride acted as a full agonist ($-\log EC_{50} = 6.9 \pm 0.03$). ICS 205-930 (3 μM) antagonized competitively responses to 5-HT, 5 methoxytryptamine, (**R,S**)- and (**S**)-zacopride and 5-carboxamidotryptamine yielding $-\log K_B$ estimates ranging from 6.1–6.5.

4 It is concluded that two different 5-HT receptors mediate excitatory neuronal responses in the guinea-pig ileum. 5-HT_3 receptors mediate the second phase of the biphasic concentration-response curve, whereas a receptor with properties distinct from the 5-HT_1-like, 5-HT_2 and 5-HT_3 subtypes mediates the initial phase of the concentration-response curve. This receptor, which exhibits a close similarity to the 5-HT_4 subtype is: (1) stimulated by 5-methoxytryptamine but not 2-methyl-5-HT; (2) stimulated selectively by certain substituted benzamides; (3) recognizes the optical isomers of zacopride and (4) is blocked by relatively high concentrations ICS 205-930 ($pK_B = 6.0$–6.5) but not ondansetron, granisetron, quipazine or N-methyl-quipazine.

Introduction

Gaddum & Picarelli (1957) showed that directly and indirectly mediated responses to 5-hydroxytryptamine (5-HT) receptors evoked contractions of guinea-pig ileum. They termed these receptors D and M, respectively. The indirect responses are sensitive to atropine and involve the release of acetylcholine (Clarke *et al.*, 1989). 5-HT receptors have been most recently classified as 5-HT_1-like, 5-HT_2 and 5-HT_3 (Bradley *et al.*, 1986). The 5-HT_3 subtype, which corresponds to the M receptor (see Clarke *et al.*, 1989 for discussion), is selectively antagonized in the guinea-pig ileum by ICS 205-930 ($pA_2 = 8.0$, Richardson *et al.*, 1985), renzapride (BRL 24924; $pA_2 = 7.3$, Sanger, 1987), granisetron (BRL 43694; $pA_2 = 7.8$, Sanger & Nelson, 1989), zacopride ($pA_2 = 8.5$, Smith *et al.*, 1988) and ondansetron (GR 38032F; $pA_2 = 7.0$, Butler *et al.*, 1988).

Studies on guinea-pig isolated longitudinal ileal muscle strips (Kilbinger & Pfeuffer-Freidrich, 1985; Buchheit *et al.*, 1985) have revealed a biphasic concentration-response curve to 5-HT which is sensitive to tetrodotoxin (TTX) and it has been suggested that two different 5-HT receptors may modulate excitatory neuronal activity. The second component of the biphasic concentration-response curve to 5-HT is due to stimulation of 5-HT_3 receptors but the receptor mediating the initial, high potency, phase of the curve has not been extensively characterized. The initial phase, however, has been shown to be insensitive to blockade by ICS 205-930 (up to 1 μM), granisetron and ondansetron (Buchheit *et al.*, 1985; Sanger & Nelson, 1989; Butler *et al.*, 1985) confirming the lack of involvement of 5-HT_3 receptors. Similar experiments undertaken in the presence of methysergide (Costa & Furness, 1976; Buchheit *et al.*, 1985) indicate that the responses at the initial, high potency phase appear to be unrelated to stimulation of 5-HT_1-like or 5-HT_2 receptors.

Taken together the data on the unstimulated ileum (Buchheit *et al.*, 1985) suggest that a 5-HT receptor which acts to modulate excitatory neuronal activity, is present in the ileum but is distinct from 5-HT_1-like, 5-HT_2 or 5-HT_3 subtypes as currently defined (Bradley *et al.*, 1986; Myelcharane, 1989). The aim of the present study was to examine this 'orphan' 5-HT receptor on the isolated, quiescent ileum of the guinea-pig.

Methods

Portions of ileum (dissected 1.5 cm distal to the ileocaecal junction) were removed from male, Dunkin-Hartley guinea-pigs (350–400 g) which had been killed by CO_2 asphyxiation.

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Whole segments of ileum were gently flushed intraluminally with warm Tyrode solution (pH 7.4, 37°C) and prepared by the method described by Edinburgh Staff (1968). The composition of the Tyrode solution was (mM): NaCl 136.9, KCl 2.7, $MgCl_2 . 6H_2O$ 1.2, NaH_2PO_4 1.2, glucose 10.0, $NaHCO_3$ 25.0 and $CaCl_2 \cdot 6H_2O$ 2.5. It was continually gassed with 5% O_2/95% CO_2. Methysergide was added at a concentration of 1 μM as described by Smith et al. (1988) in order to exclude any potential effects of 5-HT_1-like or 5-HT_2 receptor stimulation. Portions (1.5 cm) of intact ileum were placed in Tyrode solution under 1.0 g tension and allowed to equilibrate for 60 min. During this period the bathing solution was replaced every 15 min. Preliminary experiments (R.M. Eglen, unpublished observations) showed that the tissues responded with an initial contracture when exposed to methysergide. The contracture did not persist and the baseline tension was re-attained within 5 min. The inclusion of 1 μM methysergide did not significantly affect the potency of 5-HT, a finding which is in agreement with that of Buchheit et al. (1985). However, all experiments were conducted in the presence of methysergide because Costa & Furness (1972) have demonstrated the converse.

Ileal responses were measured by determining changes in isometric tension (mg), with a Hugo Sachs K30 force transducer and a Graphtec-Watanabe Linearecorder WR3101.

In all experiments, tissues were exposed to 50 mM KCl for 3 min to obtain an estimate of the maximal size of contraction of the preparation (4.81 $\pm$ 0.06 g, mean $\pm$ s.e.mean, $n = 103$). The tissues were washed and allowed 15 min to re-attain baseline tension. Concentration-response curves were then constructed, in a non-cumulative fashion, to 5-HT (1 nM–32 μM) with an agonist exposure period of 30 s on a 5 min dose-cycle. After the final agonist exposure, the tissues were washed and left for 60 min. During this 60 min recovery period, the bathing solution was replaced every 15 min after which another agonist concentration-response curve was constructed.

In studies with antagonists, the ileum was equilibrated with a particular antagonist during the 60 min recovery period, and the antagonist was retained in the bath during construction of the subsequent concentration-response curve. Only one antagonist was exposed to each ileal segment, and parallel control studies were always undertaken to correct for changes in sensitivity of the tissue to 5-HT.

To obtain an accurate estimation of the dissociation constants of antagonists at the 5-HT_3 receptors, the technique of Fozard (1985) was employed. The preparations were placed in 10 μM 5-methoxytryptamine for the duration of the experiment, which selectively removed the initial component of the 5-HT concentration-response curve, the remaining 5-HT responses being mediated through 5-HT_3 receptors. Preliminary experiments showed that inclusion of 5-methoxytryptamine did not affect responses of the preparation to carbachol, histamine, substance P, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) or KCl (data not shown).

In separate studies, the effect of catecholamine depletion on 5-HT responses was assessed in animals that had been pretreated with reserpine (5 mg kg^{-1}, i.p.) 18 h before they were killed, a procedure that has been previously shown to remove the responses to tyramine (Eglen & Whiting, 1989).

Analysis of results

The methods used to determine agonist potencies ($-\log EC_{50}$) were similar to those described by Buchheit et al. (1985). The potencies from monophasic concentration-response curves were characterized by non-linear iterative fitting procedures (Parker & Waud, 1971) calculated with RS1 software (BBN Software Products Corp., Cambridge, MA, U.S.A.). Biphasic concentration-response curves were characterized as follows: the maxima for each phase of the curve were determined graphically, and the $-\log EC_{50}$ values were calculated with respect to the two phases, defined as follows. The $-\log EC_{50}$ values determined at the initial phase (defined as those responses occurring between 1 nM and 0.32 μM 5-HT) were denoted as $-\log EC_1$ and the maximal response denoted as max_1. The $-\log EC_{50}$ value determined at the second phase (defined as those responses occurring between 0.32 μM 5-HT and 32 μM 5-HT) was denoted as $-\log EC_2$ and the maximal response as max_2.

In some experiments, the dissociation constants for partial agonists were calculated according to the method of Barlow et al. (1967). Equiactive concentrations of 5-HT and the partial agonist were plotted in a double reciprocal fashion, with the former values plotted on the ordinate scale and latter values plotted on the abscissae. The intercept with the ordinate scale and slope of the resulting straight line were determined by linear regression. The dissociation constant (expressed as the $-\log K_A$) was calculated from the relationship:

$$-\log K_A = -\log\left(\frac{\text{slope}}{\text{intercept}}\right).$$

Antagonists

The antagonist dissociation constants were determined in two ways. Firstly, where the concentration-response curves were dextrally shifted in parallel, with no reduction in maxium response, the pA_2 values were calculated by the method of Arunlakshana & Schild (1959). The slope and the intercept of the resulting straight line with the abscissae were determined by linear regression. Secondly, in experiments where a single concentration of antagonist was used, the method employed was that described by Furchgott (1972). The $-\log K_B$ values were calculated according to the following relationship:

$$-\log K_B = -\log\left(\frac{[\text{antagonist}]}{\text{dose-ratio} - 1}\right).$$

Statistics

Statistical differences were assessed by Student's t test, with $P < 0.05$ being considered as significant; all values quoted are the mean $\pm$ s.e.mean from 6–10 experiments.

Drugs used

The following compounds were synthesized in the Institute of Chemistry, Syntex Research: ($\pm$)-pindolol, atenolol, BRL 24682 (4-amino-N(N-methyl-8-azabicyclo[3.2.1]-5-chloro-2-methoxy benzamide), renzapride (BRL 24924 ($\pm$)-endo-4-amino-5-chloro-2-methoxy-N-(1-azabicyclo[3.3.1]non-4-yl) benzamide), granisetron (BRL 43694; N-endo-9-methyl-9-azabicyclo [3.3.1] non-3-yl)-1-methyl-indazole-3-carbexamide), 5-carboxamido-tryptamine, N,N-dipropyl-5-carboxamido-tryptamine, ondansetron (GR 38032F, 1,2,3,9-tetrahydro-3-[(methyl-imidazol-1-yl)methyl]-9-methyl-4H-carbazol-4-one), GR 43175 (3-[2-dimethyl-amino]ethyl-N-methyl-1H-indole-5-methane- sulphonamide), N-methylquipazine, rauwolscine, (**R,S**)-zacopride, (**S**)-zacopride and (**R**)-zacopride. Reserpine (Serpasil) was purchased from Ciba-Geigy. Methysergide was generously donated by Sandoz, Research Institute, N.J., U.S.A. ICS 205-930 ((3α-tropanyl)-1H-indole-3-carboxylic acid ester), 8-OHDPAT (8-dihydroxydiphenylaminotetralin), sulpiride, metoclopramide and quipazine were purchased from Research Biochemicals Ltd. 5-Methoxytryptamine was obtained from Aldrich, and the remaining compounds were obtained from Sigma Chemical Co. Ltd.

Results

Studies conducted in the presence of 5-HT_1-like and 5-HT_2 receptor blockade

The contractile responses to 5-HT were rapid in onset and accompanied by a fade phenomenon, particularly at high

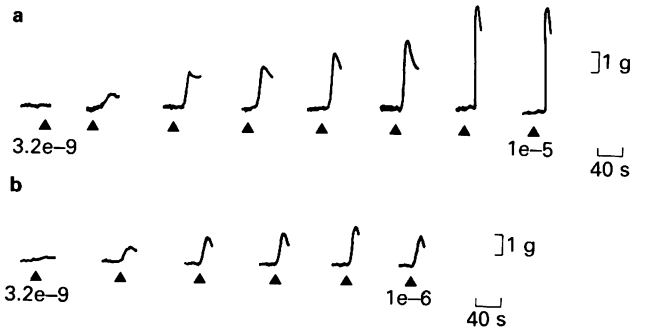

Figure 1 Recordings of representative responses to 5-hydroxytryptamine (5-HT) in the segments of whole ileum from guinea-pig. Shown are responses to 5-HT in the absence (a) or presence (b) of 0.1 μM ICS 205-930. Methysergide (1 μM) was present throughout each experiment. Concentrations of 5-HT were added, at points indicated by the closed triangles, in 3 fold molar incremental increases in concentration. The exposure period was 30 s.

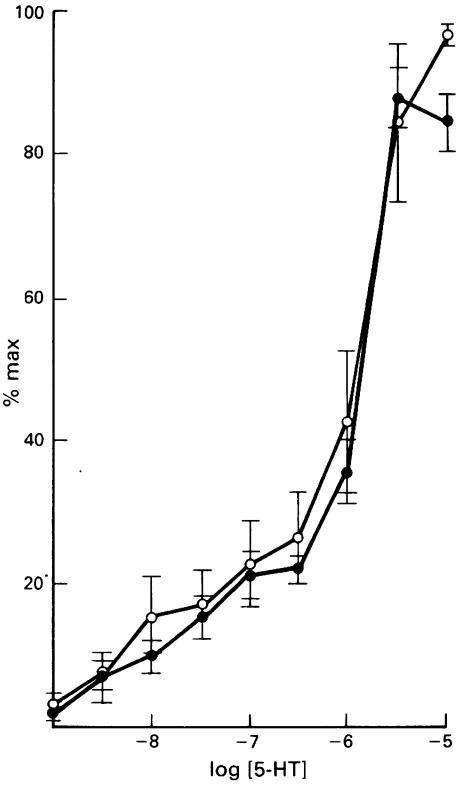

Figure 2 Concentration-response curves to 5-hydroxytryptamine (5-HT) in segments of whole ileum: (○) initial concentration-response curve; (●) second curve to 5-HT, after an interval of 60 min. Values are mean, from 6–10 preparations with the vertical bars indicating s.e.mean.

(>1 μM) concentrations (Figure 1a). The concentration-response curve to 5-HT was biphasic, consisting of an initial phase occurring between 5-HT concentrations of 3.2 nM and 0.32 μM and a second phase which occurred between 1 μM and 32 μM 5-HT. The $-\log EC_1$ value for 5-HT was 7.15 ± 0.08, and the $-\log EC_2$ value was 5.32 ± 0.03. The maximal responses at each phase were: $max_1 = 33.4 \pm 0.02\%$; $max_2 = 100\%$. No evidence of desensitization was evident with a 60 min recovery period between consecutive 5-HT concentration-response curves. In time control experiments, the first concentration-response curves exhibited a mean $-\log EC_1$ of 7.20 ± 0.11 and a mean $-\log EC_2$ of 5.35 ± 0.08, whereas the second concentration-response curve exhibited a $-\log EC_1$ of 7.23 ± 0.06 and a $-\log EC_2$ of 5.29 ± 0.11 (Figure 2). The maximal responses of either phase were also similar in the two curves.

The data obtained with 5-HT in the absence and presence of various antagonists are summarised in Tables 1 and 2. The effect of tetrodotoxin, which inhibits neuronal depolarization by blocking sodium channels (Gershon, 1967) and morphine, which inhibits acetylcholine release (Ganatra *et al.*, 1979), were studied. In the presence of tetrodotoxin (1 μM), the responses to 5-HT in the initial phase of the curve were abolished (Table 1). However, the second phase of the concentration-response curve was shifted slightly to the right but the maximum response was greatly reduced (Table 2). Similar results were

Table 1 Effect of antagonists on 5-hydroxytryptamine (5-HT) responses occurring in the initial phase of the concentration-response curve

	Control		*+ Antagonist*	
Antagonist	$-\log EC_1$	$\%\ max_1$	$-\log EC_1$	$\%\ max_1$
TTX (1 μM)	7.52 ± 0.03	36 ± 3	abolished	
Morphine (10 μM)	7.38 ± 0.11	22 ± 4	abolished	
Atropine (0.1 μM)	7.51 ± 0.06	43 ± 3	7.35 ± 0.10	12 ± 2^b
Atropine (1 μM)	7.62 ± 0.08	25 ± 8	abolished	
Physostigmine (0.1 μM)	7.83 ± 0.11	28 ± 5	7.52 ± 0.18^a	90 ± 18^b
Propranolol (1 μM)	7.48 ± 0.09	38 ± 4	7.35 ± 0.13	35 ± 2
Atenolol (1 μM)	7.53 ± 0.13	35 ± 5	7.52 ± 0.09	36 ± 7
Pindolol (1 μM)	7.61 ± 0.04	36 ± 2	7.59 ± 0.11	38 ± 4
ICI 118551 (1 μM)	7.55 ± 0.07	38 ± 7	7.62 ± 0.14	40 ± 5
Phenoxybenzamine (0.1 μM)[c]	7.54 ± 0.06	38 ± 5	abolished	
Phentolamine (1 μM)	7.52 ± 0.09	38 ± 9	7.43 ± 0.11	34 ± 8
Prazosin (1 μM)	7.55 ± 0.04	35 ± 4	7.48 ± 0.03	34 ± 5
Rauwolscine (1 μM)	7.35 ± 0.08	34 ± 5	6.85 ± 0.04^a	25 ± 3^b
Ondansetron (1 μM)	7.49 ± 0.07	36 ± 4	7.54 ± 0.13	38 ± 5
Granisetron (1 μM)	7.58 ± 0.09	34 ± 8	7.62 ± 0.11	37 ± 6
ICS 205-930 (1 μM)	7.53 ± 0.11	32 ± 6	7.44 ± 0.04	28 ± 4
Quipazine (1 μM)	7.46 ± 0.08	37 ± 9	7.38 ± 0.12	35 ± 6
N-methylquipazine (1 μM)	7.69 ± 0.04	38 ± 7	7.59 ± 0.11	36 ± 9
(R,S)-zacopride (1 μM)	7.78 ± 0.11	35 ± 4	7.32 ± 0.14	8 ± 2^b

Values are mean ± s.e.mean, $n = 6$–10.
[a] Significantly different ($P < 0.05$) from control $-\log EC_1$ value.
[b] Significantly different ($P < 0.05$) from control $\%\ max_1$ value.
[c] Phenoxybenzamine was allowed 60 min to equilibrate and the second curve to 5-HT was constructed in the presence of phenoxybenzamine.

Table 2 Effect of antagonists on 5-hydroxytryptamine (5-HT) responses occurring in the second phase of the concentration-response curve

	Control		*+ Antagonist*	
Antagonist	$-\log EC_2$	% max_2	$-\log EC_2$	% max_2
TTX (1 μM)	5.81 ± 0.06	100	5.61 ± 0.11[a]	12 ± 2[b]
Morphine (10 μM)	5.73 ± 0.08	100	5.24 ± 0.08[a]	38 ± 4[b]
Atropine (0.1 μM)	5.68 ± 0.11	100	5.18 ± 0.12[a]	34 ± 8[b]
Atropine (1 μM)	5.75 ± 0.03	100	5.32 ± 0.11[a]	18 ± 4[b]
Physostigmine (0.1 μM)	5.52 ± 0.04	100	5.43 ± 0.17	141 ± 19[b]
Propranolol (1 μM)	5.69 ± 0.08	100	5.63 ± 0.05	100
Atenolol (1 μM)	5.71 ± 0.11	100	5.73 ± 0.08	100
Pindolol (1 μM)	5.75 ± 0.04	100	5.69 ± 0.14	100
ICI 118551 (1 μM)	5.77 ± 0.07	100	5.81 ± 0.11	100
Phenoxybenzamine (0.1 μM)[c]	5.72 ± 0.08	100	5.75 ± 0.09	30 ± 4[b]
Phentolamine (1 μM)	5.73 ± 0.04	100	5.88 ± 0.06	100
Prazosin (1 μM)	5.68 ± 0.11	100	5.73 ± 0.04	100
Rauwolscine (1 μM)	5.63 ± 0.04	100	5.59 ± 0.08	100
Ondansetron (1 μM)	5.64 ± 0.14	100	abolished[a]	—
Granisetron (1 μM)	5.66 ± 0.18	100	abolished[a]	—
ICS 205-930 (0.1 μM)	5.73 ± 0.08	100	abolished[a]	
Quipazine (1 μM)	5.78 ± 0.12	100	abolished[a]	
N-methyl-quipazine (1 μM)	5.81 ± 0.18	100	abolished[a]	—
(R,S)-zacopride (1 μM)	5.68 ± 0.07	100	abolished[a]	—

Values are mean ± s.e.mean, $n = 6$–10.
[a] Significantly different ($P < 0.05$) from control $-\log EC_2$ value.
[b] Significantly different ($P < 0.05$) from control % max_2 value.
[c] Phenoxybenzamine was allowed 60 min to equilibrate and the second curve to 5-HT was constructed in the presence of phenoxybenzamine.

seen with morphine (10 μM) in that the initial phase of the concentration-response curve to 5-HT was abolished (Table 1), whereas the second phase was shifted to the right and the maximum was reduced (Table 2).

Pre-exposure of the tissues to 0.1 μM phenoxybenzamine for a 15 min period (when followed by a 45 min washout period) was without significant effect on either component of the concentration-response curve to 5-HT (data not shown). However, when phenoxybenzamine (0.1 μM) was equilibrated with the tissue for 60 min and a second concentration-response curve was constructed in its presence, the initial portion of the curve was abolished and the second phase reduced by 70%. In order to assess the effect of phenoxybenzamine on direct muscarinic receptor stimulation, identical experiments were performed with the muscarinic agonist carbachol instead of 5-HT. A parallel dextral shift in the concentration-response curve to carbachol was obtained (control $-\log EC_{50} = 6.52 \pm 0.04$; plus phenoxybenzamine $-\log EC_{50} = 5.53 \pm 0.08$).

Since phenoxybenzamine also possesses α-adrenoceptor antagonist activity, further experiments were undertaken in which different α-adrenoceptor antagonists were used: phentolamine (a non-selective adrenoceptor antagonist), prazosin (an α_1-adrenoceptor selective antagonist) and rauwolscine (an α_2-adrenoceptor selective agonist). There was no significant effect on either phase of the concentration-response curve to 5-HT established in the presence of either 1 μM phentolamine or prazosin (Table 1). In the presence of 1 μM rauwolscine, however, the initial phase of the 5-HT curve was slightly but significantly shifted to the right. The maximal response was also reduced whereas the second phase was unaffected (Tables 1 and 2). The effects of the β-adrenoceptor antagonists, propranolol and pindolol (non-selective β-adrenoceptor antagonists), atenolol (β_1-adrenoceptor selective) and ICI 118551 (β_2-adrenoceptor selective) were also studied on the 5-HT responses. At 1 μM there was no significant effect of these compounds on either phase of the 5-HT concentration-response curve (Tables 1 and 2).

Atropine (0.1 μM) markedly affected the concentration-response curve to 5-HT, in that both phases were shifted dextrally and the maximal responsed were reduced (Figure 3). Atropine at 1 μM completely abolished the initial component and markedly reduced the second component. Conversely, physostigmine (0.1 μM) potentiated the maxima of both phases of the concentration-response curve to 5-HT (Figure 4). The potency of 5-HT on the initial phase was slightly but significantly reduced, whilst no effect was seen on the second phase (Tables 1 and 2).

The concentration-response curve to the selective 5-HT_3 agonist, 2-methyl-5-HT was uniphasic ($-\log EC_{50} = 5.4 \pm 0.03$), and the maximum response was not significantly different from that attained with 10 μM 5-HT. The concentration-response curve to 5-methoxytryptamine was also uniphasic ($-\log EC_{50} = 7.1 \pm 0.08$), and the maximum response (35 ± 5%) was not significantly different from that attained by 1 μM 5-HT. The responses to 5-methoxytryptamine were abolished in the presence of 0.1 μM atropine.

Maximal responses to 2 methyl 5-HT were reduced by 80 ± 5%. Conversely, in the presence of physostigmine, the maxima, although not the potency, of responses to 5 methoxytryptamine were enhanced to 63 ± 8% of the 5-HT (10 μM)

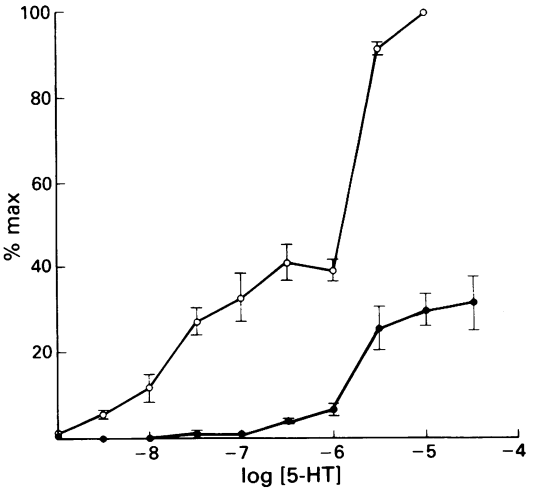

Figure 3 Concentration-response curves to 5-hydroxytryptamine (5-HT) in the absence (○) or presence of 0.1 μM (●) atropine. Values are mean, from 6–10 preparations, with vertical bars indicating s.e.mean.

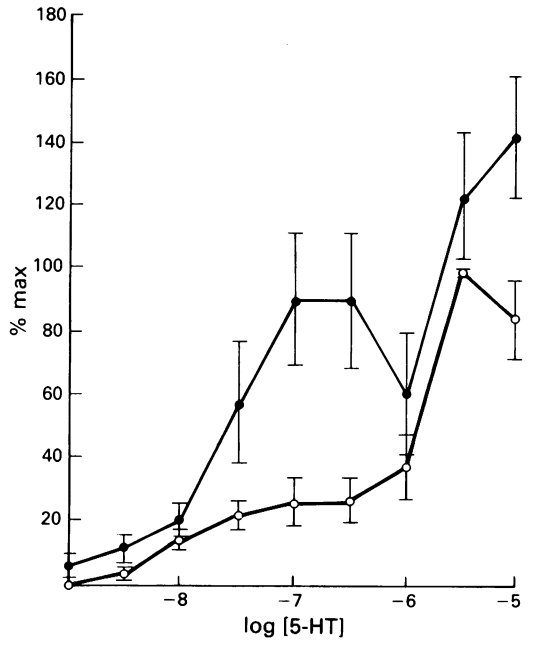

Figure 4 Concentration-response curves to 5-hydroxytryptamine (5-HT) in the absence (○) and presence (●) of 0.1 μM physostigmine. Values are mean, from 6–10 preparations, with vertical bars indicating s.e.mean.

maximal response. Furthermore, the maximal response to 2 methyl 5-HT was increased to 113 ± 4% of the maximal 5-HT (10 μM) response by physostigmine. Ondansetron, quipazine, N-methyl quipazine and granisetron did not affect the responses to 5-HT over the initial component of the concentration-response curve, whereas they antagonized the second phase of the curve. These data are shown in Tables 1 and 2. Similar results were observed in the presence of 0.1 μM ICS 205-930. However, at higher concentrations of ICS 205-930 (> 1 μM), the initial phase of the curve to 5-HT was dextrally shifted in a non-parallel fashion.

The effects of (R,S)-zacopride (10 nM–1 μM) were similar to ICS 205-930, in that the second component of the concentration-response curve was abolished, and, in addition, concentration-dependent reductions in the initial portion of the 5-HT concentration-response curve were also observed (Figure 5). During the 60 min exposure of the tissues to zacopride (0.1–10 μM), a contracture of the tissues was observed which was maximal after 5 min and declined to baseline tension levels after 40 min. This effect was not observed

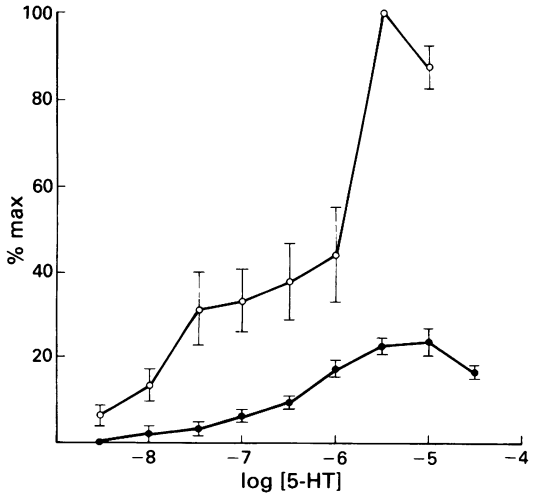

Figure 5 Concentration-response curves to 5-hydroxytryptamine (5-HT) in the absence (○) and presence (●) of 0.1 μM (R,S)-zacopride. Values are mean, from 6–10 preparations, with vertical bars indicating s.e.mean.

with either ICS 205-930, ondansetron, quipazine, N-methylquipazine or granisetron at the concentrations studied (1 nM–10 μM) (Table 3). The effects seen with (R,S)-zacopride may not represent receptor blockade, but desensitization since (R,S)-zacopride acts as an agonist at the site mediating the initial component (see below).

In the presence of 10 μM 5-methoxytryptamine and 1 μM methysergide, the concentration-response curve to 5-HT was uniphasic with no evidence of an initial component. Under these conditions, pA_2 values versus 5-HT were obtained with ICS 205-930, ondansetron, granisetron, (R,S)-zacopride, (R)-zacopride and (S)-zacopride (Table 4). All compounds exhibited Schild slopes which were not significantly different from unity, with the exception of (R,S)-zacopride. At concentrations of granisetron above 0.32 μM, the concentration-response curves to 5-HT were shifted dextrally in a non-parallel fashion, and were accompanied by a depression in the maximum. When the unity constraint was imposed, the following rank order of dissociation constants was found: (S)-zacopride > granisetron > (R,S)-zacopride = ICS 205-930 > (R)-zacopride > ondansetron (Table 4).

Table 3 Potencies ($-\log EC_{50}$) and maximal responses (% max) relative to 5-hydroxytryptamine (5-HT) of agonist eliciting contractile responses of guinea-pig ileum

Agonist	$-\log EC_{50}$	% max
5-HT	7.5 ± 0.08	1.0
5-methoxytryptamine	7.0 ± 0.03	1.0
Renzapride	7.0 ± 0.06	0.8
(S)-zacopride	6.9 ± 0.03	1.0
(R,S)-zacopride	6.3 ± 0.12	0.8
(R)-zacopride	5.7 ± 0.08	0.4
5-carboxamidotryptamine	6.1 ± 0.12	0.6
BRL 24682	5.9 ± 0.11	0.4
Metoclopramide	5.5 ± 0.12	0.6
Sulpiride	>4.0	—
2-methyl-5-HT	inactive	—
N,N,-dipropyl-5-carboxamidotryptamine	inactive	—
GR 43175	inactive	—
B-OHDPAT	inactive	—
Ondansetron	inactive	—
ICS 205-930	inactive	—
Granisetron	inactive	—
Quipazine	inactive	—
N-methyl-quipazine	inactive	—

Values are mean ± s.e.mean, n = 4–8. All agonists studied between 1 nM–10 μM. All studies conducted in the presence of 1 μM methysergide and 1 μM ondansetron to exclude 5-HT_1-like, 5-HT_2 and 5-HT_3 receptor function.

Table 4 pA_2 values and Schild slopes of antagonists at 5-HT_3 receptors in guinea-pig ileum

Antagonist	pA_2	*Slope*	pK_B
ICS 205-930	7.62 ± 0.10	0.98 ± 0.12	7.61 ± 0.05
Ondansetron	6.95 ± 0.10	0.88 ± 0.11	6.91 ± 0.04
Granisetron[a]	7.84 ± 0.07	1.09 ± 0.08	7.90 ± 0.04
(R,S)-zacopride	7.86 ± 0.24	1.23 ± 0.15[b]	7.64 ± 0.11
(R)-zacopride	7.15 ± 0.09	1.18 ± 0.14	7.27 ± 0.06
(S)-zacopride	7.96 ± 0.14	1.15 ± 0.16	8.11 ± 0.06

Values are mean ± s.e.mean, n = 8–16. All experiments were conducted in the presence of 10 μM 5-methoxytryptamine in order to desensitize selectively the initial phase of the 5-HT concentration-response curve. pK_B values are those calculated after imposing the unity constraint.

[a] At concentrations of granisetron above 0.32 μM, the maximum and slope of the concentration-response curves were reduced. Consequently, the pA_2 values were calculated with concentrations up to and including 0.32 μM.

[b] Significantly different from unity.

Table 5 Equilibrium dissociation constants ($-\log K_B$) for ICS 205-930 (3 μM) at 5-HT receptors mediating ileal contractions in the presence of 1 μM methysergide and ondansetron (1 μM)

Agonist	$-log\ K_B$
5-HT	6.31 ± 0.12
5-Methoxytryptamine	6.02 ± 0.09
(S)-zacopride	6.26 ± 0.13
(R,S)-zacopride	6.52 ± 0.14
5-Carboxamidotryptamine	6.10 ± 0.05

Values are mean ± s.e.mean, n = 4–8.

Studies conducted in the presence of 5-HT$_1$-like, 5-HT$_2$ and 5-HT$_3$ receptor blockade

In order to characterize further the initial phase of the concentration-response curve to 5-HT, the preparations were placed in Tyrode solution containing both 1 μM methysergide and 1 μM ondansetron, to exclude responses at 5-HT$_1$-like, 5-HT$_2$ and 5-HT$_3$ receptors (Buchheit *et al.*, 1985; Richardson *et al.*, 1985). Under these conditions the concentration-response curve to 5-HT was uniphasic (Figure 1b). The potency ($-\log EC_{50} = 7.2 \pm 0.12$) and maximal response (34.5 ± 6%) to 5-HT were not significantly different from the potency and maximal response to 5-HT at receptors mediating the initial phase of the concentration-response curve, when determined in the absence of ondansetron. The responses to 5-HT were highly reproducible, with no evidence of desensitization occurring between 2 consecutive concentration-response curves when established after a 60 min interval (curve 1, $-\log EC_{50} = 7.2 \pm 0.05$; curve 2, $-\log EC_{50} = 7.3 \pm 0.08$. The maximum responses of the two curves were not significantly different).

The responses to 2-methyl 5-HT were abolished in the presence of both methysergide (1 μM) and ondansetron (1 μM) whereas responses to 5-methoxytryptamine were not significantly affected ($-\log EC_{50} = 7.1 \pm 0.08$; maximum = 31 ± 4%). The potencies of 5-HT, 5-methoxytryptamine and 2-methyl 5-HT were also unaffected following reserpine pretreatment in terms of both EC_{50} and maximal responses at both phases of the concentration-response curve (data not shown).

The potencies ($-\log EC_{50}$ values) and intrinsic activities, relative to the maximum response to 5-HT (% maximum response) of a number of agonists at receptors mediating the contractile response in the presence of 1 μM ondansetron and 1 μM methysergide, are shown in Table 3. The rank order of agonist potency was 5-HT > 5-methoxytryptamine = (S)-zacopride = renzapride > (R,S)-zacopride > 5-carboxamidotryptamine > BRL 24682 > (R)-zacopride > metoclopramide ≫ sulpiride = N,N,dipropyl-5-carboxamidotryptamine = 8-OHDPAT = GR 43175 = 0. Only 5-HT, 5-methoxytryptamine and (S)-zacopride acted as full agonists. None of the 'inactive' compounds antagonized responses to 5-HT after equilibration for 60 min at a concentration of 1 μM. As (R,S)-zacopride and (R)-zacopride acted as partial agonists (Table 4), the dissociation constants ($-\log K_A$) were calculated by the method of Barlow *et al.* (1967). The $-\log K_A$ values for (R,S)- and (R)-zacopride were 6.1 ± 0.08 and 5.5 ± 0.11, respectively. The responses to (R,S)-zacopride and its isomers were abolished in the presence of 0.1 μM atropine, whereas the maximal response was significantly potentiated in the presence of 0.1 μM physostigmine (98 ± 4%). Atropine (0.1 μM) also abolished the contractile responses to all the other 'active' agonists (data not shown).

In separate experiments, the responses to 5-HT, 5-methoxytryptamine, (S)-zacopride, (R,S)-zacopride and 5-carboxamidotryptamine were studied in the absence and presence of a high concentration of ICS 205-930 (3 μM: relative to its equilibrium dissociation constant at 5-HT$_3$ receptor; see above). To eliminate completely the possibility of an interaction at 5-HT$_3$ receptors, 1 μM ondansetron was also present throughout the experiment. The concentration-response curves to the agonists were shifted dextrally in a parallel fashion by ICS 205-930. It can be seen that the $-\log K_B$ values of ICS 205-930 obtained with these agonists were very similar (Table 5). In order to study the specificity of action of ICS 205-930, the effect of 10 μM ICS 205-930 against responses to carbachol and DMPP was studied. Responses to these agonists were not significantly affected either in terms of potency or maxima (carbachol control, $-\log EC_{50} = 6.9 \pm 0.06$; in the presence of 10 μM ICS 205-930, $-\log EC_{50} = 7.1 \pm 0.11$; DMPP control, $-\log EC_{50} = 5.6 \pm 0.11$; in the presence of 10 μM ICS 205-930, $-\log EC_{50} = 5.6 \pm 0.14$).

Discussion

The present study has characterized the receptors mediating the indirectly mediated contractile response to the neuronally mediated effects of 5-HT in isolated segments of guinea-pig ileum. This preparation has been shown to exhibit a biphasic concentration-response curve to 5-HT (Fozard, 1985; Buchheit *et al.*, 1985; Butler *et al.*, 1988; Sanger & Nelson, 1989). The second portion of the curve is mediated by stimulation of 5-HT$_3$ receptors (Richardson *et al.*, 1985; Butler *et al.*, 1988; Cohen *et al.*, 1988), whereas the receptor mediating the initial phase of the concentration-response curve remains to be characterized.

The data obtained in the first series of experiments (Tables 1 and 2), in which both phases of the concentration-response curve to 5-HT were studied, are in good agreement with previous results (Buchheit *et al.*, 1985; Butler *et al.*, 1988; Sanger & Nelson, 1989). However, the lack of effect of methysergide while in agreement with data obtained by Buchheit *et al.* (1985) contrasted with that of Costa & Furness (1972) in which an inhibitory effect of methysergide was seen. The receptors mediating both phases of the biphasic concentration-effect curve appear to be neuronally located, in view of their high sensitivity to the sodium channel blocker, tetrodotoxin. The sensitivity of the 5-HT responses to morphine is in agreement with previous studies (Paton, 1957; Schaumann, 1957; Ganatra *et al.*, 1979) and is due to inhibition acetylcholine release. Pre-exposure of the preparations to phenoxybenzamine (0.1 μM) for a brief period (15 min), followed by subsequent washout, did not affect responses to 5-HT over either phase. However, in the maintained presence of phenoxybenzamine responses to 5-HT over the second phase responses to 5-HT were reduced by 70% whilst the responses over the initial phase were abolished. A reasonable explanation for the effect of phenoxybenzamine is that alkylation of postjunctional muscarinic receptors occurred which decreased the indirect effects of 5-HT. The ability of phenoxybenzamine, under similar conditions to those used in the 5-HT studies, to antagonize responses to carbachol is in agreement with this hypothesis.

The lack of effect of the α-adrenoceptor antagonists prazosin and phentolamine on the 5-HT response suggest that these effects were not due to α_1-adrenoceptor blockade. A small, but significant, inhibitory effect was observed with rauwolscine. In addition to its well characterized α_2-adrenoceptor antagonist properties this antagonist has been shown (Kaumann, 1983; Clinschmidt *et al.*, 1985) to antagonize 5-HT responses in the bovine coronary artery and rat stomach fundus. The 5-HT responses of both phases were unaffected in the presence of either propranolol, pindolol, atenolol or ICI 118551, suggesting that β_1- or β_2-adrenoceptors do not participate in the responses to 5-HT. Therefore, the receptors mediating both phases of the concentration-effect curve to 5-HT may differ from those shown to mediate positive chronotropic responses in guinea-pig atria (Eglen & Whiting, 1989). In this latter preparation 5-HT responses are non-competitively antagonized by pindolol and atenolol.

The present studies on the second component of the 5-HT concentration effect curve are in accord with the literature in

that this component appears to be mediated by 5-HT_3 receptor stimulation. The 5-HT_3 antagonists (ICS 205-930, ondansetron, granisetron, quipazine and N-methyl-quipazine) all antagonized the second phase of the concentration-response curve to 5-HT as well as responses to the 5-HT_3 agonist, 2-methyl 5-HT. Conversely, the inclusion of 5-methoxytryptamine in the Tyrode solution selectively abolished the initial phase of the concentration-response curve to 5-HT in agreement with a previous report (Fozard, 1985). The curve to 5-HT became monophasic in the presence of 5-methoxytryptamine, allowing unambiguous estimations of the effect of antagonists at the remaining 5-HT_3 receptors. The pA_2 values obtained under these conditions for ICS 205-930, ondansetron, granisetron, (**R,S**)-zacopride and its constituent enantiomers were in good agreement with other values in guinea-pig ileum reported in the literature (see Introduction for references and Fozard, 1988 for review). The present study shows (**S**)-zacopride to about 10 fold greater than (**R**)-zacopride, in terms of affinity at the 5-HT_3 receptor (8.11 versus 7.27, respectively). These values are similar to the pA_2 estimate for (**R,S**)-zacopride in guinea-pig ileum (Smith *et al.*, 1988) but the isometric ratio is considerably larger than that reported in binding studies in guinea-pig ileum with [^{3}H]-zacopride (**R/S** ratio = 1.4; Pinkus *et al.*, 1989a). Isometric ratios for zacopride (**R/S**) of 28, 42, 6 and 12 were obtained in rabbit ileum, vagus nerve, rat ileum and rat brain respectively (Pinkus *et al.*, 1989a,b).

The data are in agreement with the hypothesis that the biphasic nature of the concentration-response curve to 5-HT is mediated through two distinct receptors, the second phase being mediated by a 5-HT_3 receptor. The finding that the initial phase of the concentration-effect curve to 5-HT is unaffected by ondansetron, granisetron, quipazine and N-methyl quipazine show that, in this respect, they are more selective 5-HT_3 antagonists than either (**R,S**)-zacopride or ICS 205-930. Higher concentrations (0.1 μM–10 μM) of these latter two compounds also inhibited the initial phase of the 5-HT concentration-response curve suggesting effects independent of an interaction at the 5-HT_3 receptor.

Stimulation of the receptor mediating the initial phase of the concentration-response curve appears to be due exclusively to an enhancement of acetylcholine release. The antagonism of the response by atropine, morphine and phenoxybenzamine and its potentiation by physostigmine is in support of this proposal and with previous reports (Buchheit *et al.*, 1985; Sanger & Nelson, 1989). The second, 5-HT_3 receptor-mediated phase of the concentration-response curve also involves the release of acetylcholine, in view of the sensitivity of 5-HT and 2-methyl 5-HT responses to atropine, physostigmine and phenoxybenzamine (this study; Cohen *et al.*, 1985; Fox & Morton, 1989). A residual component of the 5-HT_3-mediated response, which was observed in the presence of atropine (this study), may involve the release of a second neurotransmitter. Buchheit *et al.* (1965) have proposed this to be substance P, although this has been disputed.

The inclusion of 1 μM ondansetron in the Tyrode solution, to inhibit 5-HT_3 function, enabled the effects of agonists to be selectively measured at the 5-HT receptor mediating the initial phase of the curve. The responses to 5-HT, 5-methoxytryptamine, (**S**)-zacopride, (**R,S**)-zacopride and 5-carboxamidotryptamine were competitively antagonized by ICS 205-930 with similar $-\log K_B$ values, indicating that these agonists interacted at the same receptor site. The concentration of ICS 205-930 required to elicit a significant dextral shift (3 μM), whilst relatively high, was specific in that no effect was observed directly at muscarinic or nicotinic receptors, as judged by the lack of effect on the responses to carbachol and DMPP.

The 5-HT receptor mediating the initial phase of the biphasic concentration-effect curve recognized optical isomers of zacopride i.e., (**S**)-zacopride was more potent than (**R**)-zacopride as an agonist. This stereoselectivity was the same for antagonism of the 5-HT_3 receptor. In terms of affinity, (**R,S**)-zacopride was approximately 100 fold more selective for ileal 5-HT_3 receptors (pA_2 = 7·9–8·5; Smith *et al.*, 1988; Cohen *et al.*, 1989; this study), in comparison to the receptor mediating the initial phase of the response curve to 5-HT. The structurally related benzamides, BRL 24682 and metoclopramide were weaker agonists whilst other 5-HT_3 antagonists, ondansetron, ICS 205-930, granisetron, quipazine and N-methyl-quipazine were inactive as agonists. These data indicate that the agonist actions of (**R,S**)-zacopride, renzapride, BRL 24682 and metoclopramide were independent of their 5-HT_3 antagonist properties. In addition, 5-methoxytryptamine, a potent agonist at receptors mediating the initial phase, is devoid of 5-HT_3 agonist and antagonist properties (Fozard, 1985; this study).

Taken together these data further suggest that the site mediating the first phase of the biphasic curve to 5-HT is distinct from the 5-HT_3 receptor. The differential affinity of ICS 205-930 at the 5-HT_3 receptor (7.6) and at the former site (6.3) is in accordance with this hypothesis. Since this site was also insensitive to methysergide it appeared to be different from 5-HT_1-like and 5-HT_2 receptors. The contractile response to 5-carboxamidotryptamine, but not to N,N-dipropyl-5-carboxamidotryptamine, observed at the receptors mediating the initial phase, also suggests that the receptor is dissimilar to the 5-HT_1-like receptor. This postulate is also consistent with the lack of contractile responses with the 5-HT_1-like and 5-HT_{1A} agonists, GR 43175 and 8-OHDPAT, respectively.

The pharmacology of the receptor mediating the initial phase of the response to 5-HT is similar to the pharmacology of the 5-HT receptor which mediates an increase in the 'twitch' response of the field-stimulated guinea-pig ileum (Sanger, 1987; Craig & Clarke, 1989). This latter response is insensitive to 5-HT_1-like, 5-HT_2 and 5-HT_3 antagonists, but sensitive to high concentrations (0.3 μM and greater) of ICS 205-930 (Craig & Clarke, 1989). It should be noted that whilst the $-\log K_B$ values for ICS 205-930 observed in the present study were similar to those reported by Craig & Clarke (1989), the potency of 5-HT at receptors mediating the contractile response in the unstimulated ileum was less than in the field-stimulated ileum ($-\log EC_{50}$ values: present study, = 7.3; field stimulated tissue, = 8.5; Craig & Clarke, 1989). Potent responses to 5-HT, equivalent to the first phase of the biphasic concentration-response to 5-HT, have also been obtained in the field-stimulated guinea-pig ileum (Sanger, 1987; Craig & Clarke, 1989). 'Twitch' responses to electrical stimulation were enhanced by low concentrations (1 nM–1 μM) of 5-HT (Kilbinger & Pfeuffer-Friedrich, 1985; Sanger, 1987; Craig & Clarke, 1989) and by 5-HT_3 antagonists of the substituted benzamide class, including renzapride (Sanger, 1987), zacopride (Craig & Clarke, 1989) and cisapride (Neya *et al.*, 1985; Schuurkes *et al.*, 1985). No enhancement was obtained with ICS 205-930 (Sanger, 1987) or granisetron (Sanger & Nelson, 1989). (**R,S**)-zacopride was a partial agonist at receptors in the quiescent ileum, but a full agonist in the field-stimulated ileum. These differences may reflect differences in effective receptor reserves between the two preparations rather than receptor differences *per se*.

The receptor mediating the contractile responses of the first phase is clearly not a 5-HT_1-like, 5-HT_2 or 5-HT_3 receptor, when defined by the criteria of Bradley *et al.* (1986). It most resembles the putative 5-HT_4 receptor recently described by Dumuis *et al.* (1988, 1989) which is present in both guinea-pig hippocampus and mouse colliculi neuronal culture. It should be noted that the term 5-HT_4 receptor has not been officially recognized by the Serotonin Club Receptor Nomeclature Committee (July 1990). The stimulation of this latter receptor leads to an increase in adenylate cyclase activity, a response which is insensitive to 5-HT_1-like, 5-HT_2 or 5-HT_3 ligands, but is antagonized by high concentrations of ICS 205-930 (pK_i values = 6.0 and 6.3 in mouse embryo colliculi neuronal cultures and guinea-pig hippocampus slices, respectively; Dumuis *et al.*, 1988). In addition, 5-methoxytryptamine (Dumuis *et al.*, 1988) and renzapride (Dumuis *et al.*, 1989) are also potent

agonists in these preparations. Since the receptor mediating the initial phase of the responses to 5-HT in the quiescent ileum also exhibits this profile it is our contention that 5-HT_4 receptors also mediate this response.

In conclusion, indirect excitatory responses to 5-HT in whole segments of guinea-pig ileum appear to be mediated by two distinct receptors. The pharmacological profiles of these receptors suggest stimulation of 5-HT_4 and 5-HT_3 subtypes. Definitive evidence for this hypothesis, however, awaits the development of potent and selective 5-HT_4 receptor antagonists.

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