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Identification and distribution of 5-HT_3 recognition sites in the rat gastrointestinal tract

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1 Tritiated derivatives of the potent and selective 5-HT_3 receptor antagonists GR65630 and LY278584 were used to identify 5-HT_3 recognition sites in the rat gastrointestinal tract.

2 Binding studies were carried out in homogenates of the rat oesophagus, the cardia, fundus, body and antrum of the stomach, regions of the small intestine, caecum and large intestine. The specific binding of a single concentration of GR65630 (0.5 nM) defined by granisetron (10 μM) in these areas indicated that the density of 5-HT_3 recognition sites varied from 2.4 ± 1.0 to 10.1 ± 1.0 fmol mg^{-1} protein.

3 Saturable binding of [^{3}H]-GR65630 could only be demonstrated in the terminal regions of the small intestine (B_{max} in the range of 13.83 ± 4.54–21.19 ± 0.89 fmol mg^{-1} protein; mean ± s.e.mean) and of high affinity (K_d in the range of 0.42 ± 0.18–0.79 ± 0.24 nM). Use of [^{3}H]-LY278584 revealed a similar binding density (B_{max} 19.54 ± 0.26 fmol mg^{-1} protein) and affinity (K_d 1.04 ± 0.07 nM) in the terminal small intestine.

4 Binding of [^{3}H]-GR65630 and [^{3}H]-LY278584 to the terminal region of the small intestine was inhibited by 5-HT_3 receptor ligands ondansetron and S-zacopride (and 5-hydroxytryptamine), but not by 5-HT_1, 5-HT_2, catecholamine, γ-aminobutyric acid and opioid receptor ligands.

5 These data demonstrate that there are regional variations in the density of 5-HT_3 recognition sites within the rat gastrointestinal tract. Such data are relevant to the potential use of 5-HT_3 receptor ligands to modify secretory and contraction responses in the gastrointestinal system.

Keywords: 5-HT_3 recognition sites; [^{3}H]-GR65630; [^{3}H]-LY278584; rat intestine

Introduction

A neuronally located 5-hydroxytryptamine (5-HT) receptor mediating transmitter release was first identified in the guinea-pig ileum by Gaddum & Picarelli (1957). Subsequent studies established that 5-HT receptors are widely distributed on peripheral nerves (see review by Richardson & Engel, 1986), and the development of selective antagonists for the 5-HT receptor subtypes has allowed characterization and designation of this neuronal receptor as the 5-HT_3 receptor (see Bradley *et al.*, 1986). The characterization was based on an antagonism by 5-HT_3 receptor antagonists such as MDL72222, ICS205-930 and ondansetron of the effects of 5-HT to induce, for example, neuronal depolarization of the isolated vagus nerve, superior cervical ganglion and the von Bezold Jarisch reflex (Fozard, 1984; Richardson *et al.*, 1985; Butler *et al.*, 1988). This initial pharmacological characterization was achieved before these 5-HT_3 receptors could be defined by radioligand binding assays. However, with the development of radiolabelled ligands such as [^{3}H]-GR65630, [^{3}H]-ICS205-930 and [^{3}H]-zacopride, 5-HT_3 receptors were identified in the brains of many species including man (Kilpatrick *et al.*, 1987; Barnes *et al.*, 1988; 1989; Waeber *et al.*, 1988), the vagus nerve and superior cervical ganglion (Kilpatrick *et al.*, 1989; Hoyer *et al.*, 1989).

However, few studies have attempted to characterize 5-HT_3 receptors in the gastrointestinal tract via radioligand binding assays. [^{3}H]-zacopride has been used at a single concentration for the tentative identification of 5-HT_3 recognition sites in certain areas of the rabbit, rat and ferret gastrointestinal tract (Pinkus *et al.*, 1989), which are associated with enteric neuronal plasma membranes (Gordon *et al.*, 1990). Such sites may provide the sites of action for 5-HT and 5-HT_3 receptor ligands to modify contraction and secretory responses in the gastrointestinal tract (Donowitz *et al.*, 1977; Cooke & Cary, 1985; Baird & Cuthbert, 1987; Costall & Naylor, 1990).

There have been no attempts to investigate systematically the presence and density of 5-HT_3 receptors throughout the whole gastrointestinal tract. Such information would be particularly helpful for identification of those regions of the tract worthy of further investigation as sites of 5-HT_3 agonist-antagonist action. In the present study we determined the distribution of 5-HT_3 recognition sites in the gastrointestinal tract of the rat.

Methods

Preparation of tissue for radioligand binding

Female Hooded-Lister rats (250–300 g, Bradford bred) were killed by cervical dislocation, and the tissues removed over ice. The gastrointestinal tract was separated as follows: oesophagus, stomach (dissected into the fundus, cardia, body and antrum), small intestine (20 cm lengths measured from the stomach), caecum (complete) and large intestine (complete). The contents of the gastrointestinal tract were removed and the tissues rinsed with ice-cold 2-[4-(2-hydroxyethyl-1-piperazinyl)]-ethane sulphonic acid (HEPES) buffer (50 mM buffered to pH 7.4 with NaOH; final Na^+ concentration was 10 mM). For most experiments the tissues were stored frozen at −20°C.

For binding studies the tissue was finely chopped with scissors and homogenized (Polytron, full speed for 10 s) in 20 volumes of ice-cold HEPES buffer and centrifuged (48,000 *g*, 10 min, 4°C). The resulting supernatant was discarded and the pellet resuspended in HEPES buffer and recentrifuged. The supernatant was discarded and the pellet resuspended in HEPES buffer at a concentration of 50 mg original wet weight ml^{-1} and kept on ice until used.

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Binding studies with [^{3}H]-GR65630

Homogenates (250 µl) of the gastrointestinal tract (or HEPES buffer for filter blanks) were added to pre-incubated (2 min, 37°C) test tubes in triplicate, containing 650 µl of competing drug or buffer (containing appropriate drug-vehicle) and 100 µl [^{3}H]-GR65630 (final concentration 0.47–0.93 nM for competition studies or a range of concentrations between 0.1–5 nM for saturation studies). The tubes were incubated for 30 min at 37°C, before termination by rapid filtration through pre-wet (0.01% v/v polyethyleneimine in HEPES buffer) Whatman GF/B filters. The filters were immediately washed twice with 9.6 ml of HEPES buffer at room temperature (wash time 8 s). Filter discs were placed in scintillation vials and covered with 10 ml of 'Ultima Gold' scintillant (Packard), and left for dark adaption for 6 h before liquid scintillation spectroscopy (efficiency approximately 47%). Protein in the homogenate was measured by the Bio-Rad Coomassie Blue method (Bradford, 1976), with bovine serum albumin used as standard.

Binding studies with [^{3}H]-LY278584

Binding studies with [^{3}H]-LY278584 were carried as described above for [^{3}H]-GR65630, except that the final concentration of [^{3}H]-LY278584 was 0.43–0.58 nM for competition studies, or a range of concentrations between 0.05–5 nM for saturation studies. Binding assays were terminated with rapid filtration as above, but ice-cold buffer was used for washing the filters.

Expression of results

Estimates of equilibrium binding parameters (K_d and B_{max}) were obtained by non-linear regression analysis from the equation $y = B_{max}\ (L/(L + K_d))$ and the KaleidoGraph programme (Synergy Software, 1990); where L = free ligand concentration.

Compounds

Atropine sulphate (Sigma), dopamine hydrochloride (Sigma), granisetron hydrochloride (Glaxo), GR65630 hydrochloride (3- (5-methyl-1H-imidazole-4-yl) -1- (1-methyl-1H-indol-3-yl) -1-propanone.HCl) (Glaxo), hexamethonium hydrobromide (Sigma), 5-hydroxytryptamine maleate (5-HT, Sigma), 5-methoxytrptamine hydrochloride (Sigma), methysergide maleate (Sandoz), ondansetron hydrochloride (Glaxo), nicotine hydrogen tartrate (British Drug Houses), prazosin hydrochloride (Pfizer), pentazocine hydrochloride (Sigma), spiperone (Janssen) and (S)-zacopride hydrochloride (A.H. Robins) were prepared in distilled water and diluted with HEPES buffer as required. γ-Amino-n-butyric acid (Sigma) and noradrenaline bitartrate (Sigma) were dissolved in the minimum quantity of glacial acetic acid and made up to volume with distilled water (pH adjusted to 6–7 with sodium bicarbonate). Ritanserin (Research Biochemicals Inc.) was dissolved in the minimum quantity of methanol and made up to volume with distilled water. [^{3}H]-GR65630 (79.9 Ci mmol^{-1}, NEN) and [^{3}H]-LY278584 maleate (1-methyl-*N*-(8-methyl-8-azabicylo[3.2.1]oct-3-yl)-1*H*-indazole-3-carboxamide) (84 Ci mmol^{-1}, Amersham) were supplied in ethanol and diluted in HEPES buffer.

Results

Determination of specific binding of [^{3}H]-GR65630 and [^{3}H]-LY278584 in the rat gastrointestinal tract

In preliminary experiments, granisetron (10 µM) was found to compete for [^{3}H]-GR65630 (0.5 nM) binding to regions of the gastrointestinal tract. However, the use of the single concentration of ligand revealed marked differences between regions in the percentage inhibition of [^{3}H]-GR65630 binding. For example, 10 µM granisetron inhibited 43–63% of the total binding of [^{3}H]-GR65630 in the small intestine, whereas lower inhibition values of 14 to 36% were recorded from preparations of the oesophagus, stomach and caecum (Table 1).

The preliminary indications of specific [^{3}H]-GR65630 binding to regions of the small intestine were confirmed in subsequent studies where [^{3}H]-GR65630 (0.1–5 nM) displayed saturable specific binding (defined by 10 µM granisetron) in homogenates prepared from the terminal regions (40–60, 60–80 and 80–100 cm segments) of the small intestines. Non-linear regression analysis of the data showed that [^{3}H]-GR65630 binding was of high affinity and to a single population of receptors (K_d in the range of 0.42 ± 0.18 to 0.79 ± 0.24 nM; mean ± s.e.mean; maximum binding density, B_{max} 13.83 ± 4.54 to 21.19 ± 0.89 fmol mg^{-1} protein with r values of 0.92–0.95, Table 2 and Figure 1a). The use of [^{3}H]-LY278584 revealed a similar binding density (B_{max} 19.54 ± 0.26 fmol mg^{-1} protein) and affinity (K_d 1.04 ± 0.07 nM, r = 0.98 ± 0.01) in the terminal 80–100 cm segment of the small intestine (Table 2).

However, with preparations of the oesophagus, the four regions of stomach, the first 40 cm of the small intestine, the caecum and large intestine, non-linear regression analysis indicated that the 'specific binding' of [^{3}H]-GR65630 was not saturable and did not give good curve fits (Table 2 and Figure 1b).

Specificity of the binding of [^{3}H]-GR65630 (0.47–0.93 nM) and [^{3}H]-LY278584 (0.43–0.58 nM) to rat small intestine

In the 80–100 cm section of small intestine which had the highest density of binding sites, the 5-HT$_3$ receptor antagonists granisetron, ondansetron and (S)-zacopride competed for the binding sites with p*Ki* values in the nanomolar range (Table 3). 5-Hydroxytryptamine also effectively competed for the binding site (p*Ki* 6.15–6.93), whereas methysergide, ritanserin and 5-methoxytryptamine influencing respectively 5-HT$_1$/5-HT$_2$, 5-HT$_2$ and the putative 5-HT$_4$ receptor were ineffective. Similarly, compounds affecting catecholamine, acetylcholine, opioid and GABA receptors were without significant effect (Table 3).

Table 1 The 'specific' binding of [^{3}H]-GR65630 (0.5 nM) and [^{3}H]-LY278584 (0.5 nM) to homogenates of the rat gastrointestinal tract

Region	*Specific binding* (fmol mg^{-1} protein)	*Specific binding × 100 / Total binding*
	[^{3}H]-GR65630	
Oesophagus	4.5 ± 1	35 ± 4
Stomach		
fundus	3.0 ± 0.4	14 ± 3
cardia	3.2 ± 1	26 ± 4
body	3.1 ± 1	36 ± 18
antrum	2.4 ± 1	17 ± 5
Small intestine		
0–20 cm	5.3 ± 1	43 ± 11
20–40 cm	5.9 ± 1	63 ± 9
40–60 cm	8.2 ± 1	62 ± 2
60–80 cm	8.9 ± 2	62 ± 4
80–100 cm	10.1 ± 1	61 ± 3
Caecum	7.6 ± 3	36 ± 6
Large intestine	6.5 ± 1	42 ± 2
	[^{3}H]-LY278584	
Small intestine		
80–100 cm	6.2 ± 0.3	100 ± 2

'Specific' binding was defined by 10 µM granisetron. Values represent the mean ± s.e.mean obtained from 3 experiments.

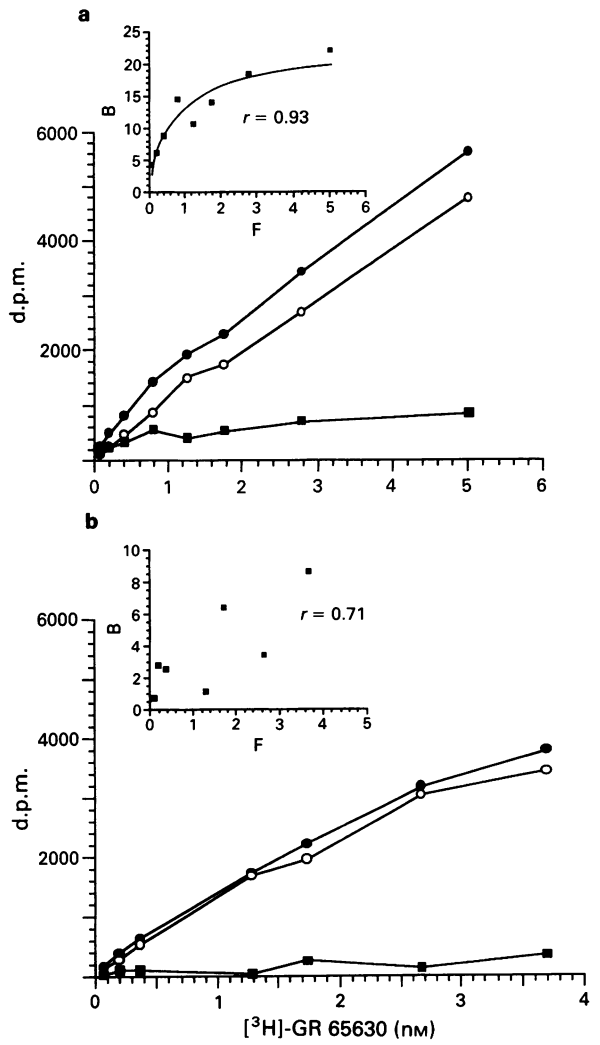

Figure 1 Representative saturation experiments with [^{3}H]-GR65630 in membranes of the rat gastrointestinal tract: (a) terminal section of small intestine and (b) oesophagus. Membranes were incubated with varying concentrations of [^{3}H]-GR65630 in the absence (● total binding) and the presence of granisetron (10 μM) (○ non-specific binding); specific binding (■). Points are means of triplicate determinations. The insets represent a non-linear regression analysis of the data; bound (B, fmol mg^{-1} protein) versus free (F, nM).

Discussion

The main purpose of this study was to determine the distribution of 5-HT$_3$ recognition sites in the gastrointestinal tract of the rat. The selective 5-HT$_3$ receptor antagonist [^{3}H]-GR65630 (Kilpatrick *et al.*, 1987) was used as the major pharmacological tool in radioligand binding studies; the 5-HT$_3$ receptor antagonist [^{3}H]-LY278584 (Robertson *et al.*, 1990) was used in more restricted experiments to provide supportive evidence for the data obtained with [^{3}H]-GR65630.

Specific binding sites defined by the use of the 5-HT$_3$ receptor antagonist, granisetron (Sanger & Nelson, 1989) were detected in the distal regions of the small intestine with both [^{3}H]-GR65630 and [^{3}H]-LY278584, the binding density being highest in the sections taken immediately before the ileo-caecal junction. The binding was most probably to a 5-HT$_3$ recognition site since the specific binding was defined by granisetron and other 5-HT$_3$ receptor antagonists, ondansetron and (S)-zacopride (Butler *et al.*, 1988; Smith *et al.*, 1988) and also 5-HT. The specificity and selectivity of the binding was further indicated by the failure of 5-HT$_1$ and 5-HT$_2$ receptor antagonists methysergide and ritanserin, and a variety of agonists and antagonists at cholinoceptor, catecholamine, opioid and GABA receptor sites to compete for the binding of [^{3}H]-GR65630 and [^{3}H]-LY278584. It is possible, if not probable, that the 5-HT$_3$ recognition sites identified in the present study may be the 5-HT$_3$ receptors that mediate changes in secretion in the rat ileum induced by 5-HT$_3$ receptor ligands (Ball *et al.*, 1988).

The presence of 5-HT$_3$ recognition sites in other parts of the rat gastrointestinal tract is less clear. Whilst the use of a single concentration of [^{3}H]-GR65630 identified 'specific' binding defined by the presence of granisetron in the oesophagus, regions of the stomach, proximal regions of the small intestine, colon and caecum, the use of a full range of concentrations and non-linear regression analysis failed to reveal saturable binding. Therefore it is difficult to demonstrate unequivocally the presence or absence of 5-HT$_3$ recognition sites in these areas of the rat gastrointestinal tract. Moreover, it is interesting that it has not been possible to characterize the 5-HT receptor mediating Cl^- secretory changes in the rat isolated colon as the 5-HT$_3$ receptor subtype (see Bunce *et al.*, 1991).

The location of 5-HT$_3$ recognition sites in different regions of the gastrointestinal tract is important to an understanding of how a pharmacological manipulation of 5-HT$_3$ receptor function might be expected to modify motility and secretion.

Table 2 Non-linear regression analysis of the specific binding of [^{3}H]-GR65630 and [^{3}H]-LY278584 to homogenates of discrete regions of the rat gastrointestinal tract

Region	B_{max} (fmol mg^{-1} protein)	K_d (nM)	r
	[^{3}H]-GR65630		
Oesophagus	NC	NC	0.81 ± 0.06
Stomach			
fundus	NC	NC	0.87 ± 0.05
cardia	NC	NC	0.87 ± 0.05
body	NC	NC	0.84 ± 0.05
antrum	NC	NC	0.71 ± 0.1
Small intestine			
0–20 cm	NC	NC	0.85 ± 0.07
20–40 cm	NC	NC	0.83 ± 0.07
40–60 cm	13.83 ± 4.54	0.42 ± 0.18	0.95 ± 0.01
60–80 cm	21.19 ± 0.89	0.79 ± 0.24	0.94 ± 0.01
80–100 cm	20.98 ± 0.97	0.47 ± 0.11	0.92 ± 0.02
Large intestine	NC	NC	0.82 ± 0.06
Caecum	NC	NC	0.68 ± 0.02
	[^{3}H]-LY278584		
Small intestine			
80–100 cm	19.54 ± 0.26	1.04 ± 0.07	0.98 ± 0.01

Values represent the mean ± s.e.mean of 3–4 separate experiments. Specific binding defined by 10 μM granisetron. NC, not calculable.

Table 3 The affinities of various compounds in competing for the binding of [^{3}H]-GR65630 (0.47–0.93 nM) and [^{3}H]-LY278584 (0.43–0.58 nM) to homogenates of the terimanl section of the rat small intestine

Compound	*[^{3}H]-GR65630* pKi	*[^{3}H]-LY278584* pKi
S-Zacopride	9.43 ± 0.21	9.52 ± 0.07
Granisetron	8.66 ± 0.11	8.54 ± 0.07
Ondansetron	8.05 ± 0.18	8.43 ± 0.09
5-Hydroxytryptamine	6.93 ± 0.07	6.15 ± 0.08
Hexamethonium	92 ± 3% TB at 0.1 μM	100 ± 5% TB at 0.1 μM
Noradrenalin	97 ± 2% TB at 0.1 μM	99 ± 3% TB at 0.1 μM
Nicotine	99 ± 5% TB at 0.1 μM	103 ± 5% TB at 0.1 μM
5-Methoxytryptamine	105 ± 3% TB at 0.1 μM	95 ± 2% TB at 0.1 μM
Pentazocine	83 ± 2% TB at 1 μM	96 ± 2% TB at 1 μM
Prazosin	87 ± 2% TB at 1 μM	105 ± 5% TB at 1 μM
Atropine	95 ± 1% TB at 1 μM	83 ± 5% TB at 1 μM
Dopamine	95 ± 6% TB at 1 μM	100 ± 6% TB at 1 μM
Spiperone	96 ± 1% TB at 1 μM	103 ± 5% TB at 1 μM
Methysergide	96 ± 6% TB at 1 μM	103 ± 6% TB at 1 μM
γ-Amino-n-butyric acid	97 ± 3% TB at 1 μM	106 ± 5% TB at 1 μM
Ritanserin	102 ± 3% TB at 1 μM	95 ± 8% TB at 1 μM

Values represent the mean ± s.e.mean of at least 3 separate experiments.
TB = total binding.

It is necessary to extend these studies to other species since 5-HT$_3$ receptor antagonists are reported to have varying effects in modifying motility and secretion in different species (see Costall & Naylor, 1990; Bunce *et al.*, 1991). Finally, the location of the 5-HT$_3$ receptors within the enteric neuronal system remains to be demonstrated.

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