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Post-synaptic 5-HT_4 receptor modulation of tachykinergic excitation of rat oesophageal tunica muscularis mucosae

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Abstract

Interaction between the 5-HT_4 receptor and cholinergic-dependent and -independent contraction of the rat oesophageal muscularis mucosae was determined. Substance P- (in the presence of atropine) and carbachol-precontracted tissue was relaxed by tryptamines and the substituted benzamides with the following rank order of potency: 5-HT > 5-methoxytryptamine > cisapride > (*R*)-zacopride > lintopride > metoclopramide, consistent with 5-HT_4 receptor activation. The response to 5-HT was not antagonized by tetrodotoxin, methysergide or ondansetron, but was shifted to the right by GR113808 ([1-[2-[methylsulphonyl)amino]ethyl]-4-piperidinyl]methyl-1-*H*-indole-3-carboxylate) in substance P- and carbachol-precontracted tissue, confirming 5-HT_4-mediated relaxation. This study shows for the first time that although 5-HT_4 receptors are involved in the modulation of cholinergic neurotransmission they can also act independently of this system modulating tachykinergic responsiveness.

Keywords: 5-HT_4 receptor; 5-HT (5-hydroxytryptamine, serotonin); Substance P; Smooth muscle; Intestinal tract; Esophagus

1. Introduction

The 5-HT receptor family is classified into four well characterized subpopulations, 5-HT_1, 5-HT_2, 5-HT_3 and 5-HT_4 (Humphrey et al., 1993). Resulting from molecular cloning studies, further subtypes of 5-HT receptors, 5-ht_{5A}, 5-ht_{5B}, 5-ht_6, and 5-ht_7 have been identified although their functional correlates remain unclear (Smith and Perker, 1995). The importance of 5-HT_4 receptors in the pharmacological treatment of a variety of pathological conditions including altered intestinal motility, functional bowel disorder, emesis and diarrhoea is now accepted (Gaster and Sanger, 1994) and the development of pharmacological tools to study this receptor is expanding rapidly. Currently the identification of 5-HT_4-mediated responses relies on a rank order potency of 5-HT ≥ 5-methoxytryptamine > cisapride > α-methyl-5-HT = (*S*)- zacopride = renzapride (Baxter et al., 1991), and antagonism by selective 5-HT_4 receptor antagonists such as GR113808 ([1-[2-[methylsulphonyl)amino]ethyl]-4-piperidinyl]methyl-1-*H*-indole-3-carboxylate; Gale et al., 1994).

Although autoradiographical studies have not been performed to determine the distribution of 5-HT_4 receptors in the gastrointestinal tract, pharmacological evidence points to their role in a range of responses in most regions of the intestinal tract and in most species. 5-HT_4 receptors mediate a contractile response in guinea-pig colonic and ileal longitudinal muscle (Wollard et al., 1994; Craig and Clarke, 1990), and a relaxation in human colonic circular muscle (Tam et al., 1994; McLean et al., 1995). In both the guinea-pig colon and ileum the effect of 5-HT_4 receptor activation appears to be mediated by cholinergic nerves. The colonic response to 5-methoxytryptamine is reduced by hyoscine (Wollard et al., 1994), while in the ileum, 5-HT_4 agonism has been shown to stimulate acetylcholine release (Kilbinger and Wolf, 1992), increase electrically stimulated cholinergic contractions (Craig and Clarke, 1990), and enhance excitatory post-synaptic potentials (Pan and Galligan, 1994) in S-type neurones (cholinergic motor or interneuronal fibres). The latter two effects were presynaptic as the responses to acetylcholine were unaffected. Like the guinea-pig ileum and colon, 5-HT_4 receptors interact with the cholinergic system of the rat tunica muscularis mucosae. This preparation differs from studies using intact guinea-pig intestine in that it is devoid of cell bodies, and it is therefore not surprising that 5-HT_4-media-

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ted effects in the rat tunica muscularis mucosae are post-synaptic, resulting in a relaxation of carbachol-precontracted tissue in the presence of tetrodotoxin (Baxter et al., 1991). Similarly, the 5-HT_4 receptor in the human colon appears to be post-synaptic (Tam et al., 1994). The exact mechanism of this post-synaptic interaction has not been fully elucidated; however, carbachol is known to activate muscarinic M_1, M_2 and M_3 receptor subtypes. In intestinal smooth muscle, muscarinic M_2 receptors mediate an inhibition of adenylate cyclase activity while muscarinic M_3 receptor activation stimulates phosphoinositide hydrolysis (Zhang and Buxton, 1993). These authors also showed that cyclic AMP-dependent protein kinase attenuated carbachol-stimulated phosphoinositide hydrolysis. As 5-HT_4-mediated relaxation of carbachol-precontracted rat oesophageal muscularis mucosae appears to be mediated by cyclic AMP, it is speculated that this relaxation is caused by a direct attenuation of phosphoinositide hydrolysis. If this is the case, 5-HT_4 receptor activation would be expected to result in functional antagonism, relaxing oesophageal smooth muscle pre-contracted by any agonist that acts through inositol trisphosphate production. This hypothesis, however, remains untested, and it is remarkable that almost all studies investigating 5-HT_4 receptors in the gastrointestinal tract have concentrated on neuromodulation or post-synaptic control of muscarinic activation.

In particular, interactions between 5-HT and tachykinergic nerves are unclear. The tachykinins are a family of important excitatory neurotransmitters in the enteric nervous system and are involved in the coordination of gastrointestinal motility (Bartho and Holzer, 1985). The tachykinins play a major pathophysiological role in inflammation and pain and are therefore likely to contribute to many intestinal diseases including gastritis, inflammatory bowel disease, allergy, nematode infection and conditions of altered sensory perception such as irritable bowel syndrome and its secondary condition, oesophagitis (Sharkey, 1992). As a result of the importance of both tachykinins and serotonin in disease it is important to fully understand their interaction. Pre-synaptic modulation of tachykinergic neurotransmission by 5-HT_4 receptors has previously been demonstrated in the guinea-pig colon, as 5-HT enhancement of electrical stimulation of non-cholinergic nerves was abolished by DAU 6285, a 5-HT_4 receptor antagonist (Kojima and Shimi, 1995). Like carbachol, substance P has been shown to stimulate inositol trisphosphate production in intestinal smooth muscle (Mallows and Bolton, 1987) and it is therefore predicted that 5-HT_4 receptor activation would post-synaptically modulate tachykinergic contraction as seen with carbachol. To explore this possibility we investigated the interaction between 5-HT and substance P in the rat oesophageal tunica muscularis. This preparation was used for three reasons: (1) it is a classic model for the study of 5-HT_4 receptor agonists and antagonists (Baxter et al., 1991); (2) post-synaptic modulation of the cholinergic system has previously been demonstrated in this organ (Baxter et al., 1991); (3) it has previously been shown to contract in response to substance P (Croci et al., 1995).

2. Materials and methods

2.1. Tissue preparation

The tunica muscularis mucosae preparation used in the present study has previously been well characterized (Croci et al., 1995; Baxter et al., 1991). Male Sprague-Dawley rats (Iffa-Crédo; 300–450 g) were killed by desanguination following a stunning blow to the head. The distal 2 cm of intrathoracic oesophagus was removed and the outer muscle layers carefully dissected by sharp dissection. The remaining muscularis mucosae/mucosal preparation was mounted in a 20 ml organ bath in its longitudinal axis with one end attached to an isometric tension transducer (Model K30; Hugo Sachs Elektronic, Germany) connected to an amplifier (Model 13-46 15-50, Gould, France) and the other anchored to the base of the bath. The tissue was bathed in a Krebs buffer and continuously gassed with 95% O_2/5% CO_2. An initial resting tension of 0.5 g was applied to the preparation and was readjusted during the initial 30 min equilibration period, washing every 10 min. Two separate series of experiments were then performed, the first to determine 5-HT_4 modulation of substance P-evoked contractions and the second to compare this to 5-HT_4 modulation of carbachol-precontracted tissue (Baxter et al., 1991). For the first series of experiments atropine (3 μM) was added to the bathing solution from the start.

2.2. Agonist response curves

After a 30 min incubation period, preparations were precontracted with submaximal concentrations of substance P (1 μM; as determined from preliminary studies) or carbachol (0.5 μM), until a reproducible response was achieved (preliminary studies showed that a sensitizing challenge with substance P was required to attain reproducibility). 10 or 15 min after precontraction with substance P or carbachol respectively 5-HT was added in a cumulative fashion. To compare the 5-HT response to various 5-HT agonists the tissue was allowed to re-equilibrate for 20 min after washout, at which time the tissue was precontracted once more and a cumulative concentration–response curve constructed for the agonist in question.

2.3. Receptor antagonist sensitivity

To determine the 5-HT receptor subtype that mediates this response, a number of specific receptor antagonists were used. In addition the possibility of a neural component to the 5-HT response was investigated using

Table 1
Potencies (pD_2) and α values for tryptamines and substituted benzamides in carbachol- and substance P-precontracted rat tunica muscularis mucosae

	pD_2		α	
	Substance P precontracted	Carbachol precontracted	Substance P precontracted	Carbachol precontracted
5-HT	8.41 (8.37–8.44)	8.04 (7.98–8.09)	1.00	1.00
5-Methoxytryptamine	8.06 (7.86–8.22)	7.69 (7.61–7.67)	1.10 ± 0.03	1.00 ± 0.00
Cisapride	7.83 (7.79–7.88)	7.60 (7.53–7.67)	0.99 ± 0.20	0.80 ± 0.01
(*R*)-Zacopride	7.14 (7.06–7.22)	6.80 (6.75–6.85)	1.05 ± 0.10	0.95 ± 0.03
Lintopride	6.93 (6.85–7.00)	6.34 (6.88–6.40)	1.06 ± 0.04	1.00 ± 0.00
Metoclopramide	6.76 (6.68–6.83)	6.19 (6.09–6.29)	0.91 ± 0.05	0.88 ± 0.06

Mean values are given with 95% intervals of confidence shown in parentheses for pD_2 values and standard errors shown for α values ($n \geq 4$).

tetrodotoxin (1 μM). For these experiments a similar protocol was followed with the exception that after the initial response to 5-HT, the tissue was equilibrated in the presence of the 5-HT receptor antagonist or tetrodotoxin prior to the construction of a second 5-HT response curve. Preliminary studies showed that two consecutive 5-HT concentration–response curves yielded similar maximal responses and EC_{50} values. The receptor antagonists used were the 5-HT_1/5-HT_2 receptor antagonist, methysergide (1 μM; Baxter et al., 1991), the 5-HT_3 receptor antagonist ondansetron (1 μM; Baxter et al., 1991) and the 5-HT_4 receptor antagonist GR113808 (10 nM; Gale et al., 1994).

2.4. Data analysis

Data were continuously collected by an acquisition package (JAD, France) which automatically determined agonist responses in grams of tension and expressed them as a percentage of the maximum response to 5-HT in the first response curve. Pooled data for each experiment were fitted to a sigmoid curve (Utistat, France) and EC_{50} values calculated. For agonists, pD_2 values ($-\log EC_{50}$) were calculated as well as α, a measure of full agonist properties, which was obtained by dividing the maximal agonist response by the maximal 5-HT response. Dose ratios were determined from single concentrations of receptor antagonists and used to calculate pK_b values using the following equation:

$$\text{Dose ratio} = EC_{50+\text{antagonist}}/EC_{50-\text{antagonist}}$$

$$pK_b = -\log([\text{receptor antagonist}]/DR - 1)$$

In each case mean values (95% confidence limits) are given. To calculate the statistical significance of differences in receptor antagonist sensitivity of carbachol- and substance P-precontracted tissues, pK_b values were recalculated for each pair of curves (response to 5-HT under control conditions and in the presence of receptor antagonist). Means for carbachol- and substance P-precontracted tissues were then compared using Students *t*-test.

2.5. Drugs and solutions

Atropine sulphate, 5-hydroxytryptamine (creatinine sulphate complex), 5-methoxytryptamine hydrochloride, carbamylcholine chloride (carbachol) and tetrodotoxin were all purchased from Sigma. Methysergide, GR113808, (*R*)-zacopride, lintopride, metoclopramide and cisapride were synthesised by the chemistry department of Synthelabo Recherche. The Krebs solution was of the following composition (mM): NaCl, 118; KCl, 4.7; $MgSO_4$, 1.18; KH_2PO_4, 1.18; glucose, 11.5; $NaHCO_3$, 24.88; $CaCl_2$,

Table 2
Potencies (pD_2) of 5-HT-mediated relaxation of substance P- and carbachol-precontracted rat tunica muscularis mucosae in the presence of various receptor antagonists

Antagonist	Substance P-precontracted tissue			Carbachol-precontracted tissue		
	Control	+ Antagonist	pK_b	Control	+ Antagonist	pK_b
Tetrodotoxin (neural blocker)	8.56 (8.44–8.68)	8.36 (8.24–8.47)	ND	ND	ND	ND
Methysergide (5-HT_1/5-HT_2)	8.57 (8.50–8.63)	8.57 (8.52–8.61)	ND	8.19 (8.13–8.25)	8.13 (8.07–8.18)	ND
Ondansetron (5-HT_3)	8.14 (8.07–8.21)	8.07 (8.02–8.13)	ND	7.88 (7.74–8.02)	7.74 (7.61–7.87)	ND
GR113808 (5-HT_4)	8.17 (8.14–8.21)	6.65 (6.62–6.69)	9.51 (9.46–9.57)	7.91 (7.89–7.94)	6.10 (6.03–6.16)	9.81 (9.78–9.91)

Antagonist concentrations were 1 μM with the exception of GR113808 (10 nM). Mean values are given with 95% intervals of confidence shown in parentheses ($n \geq 3$).

2.52. All drugs were dissolved in distilled water on a daily basis, with the exception of substance P, which was dissolved in 0.1 M acetic acid. All dilutions were made in distilled water. Substance P was stored as a stock solution at −20°C.

3. Results

3.1. Agonist responsiveness

Both substance P (1 μM) and carbachol (0.5 μM) evoked reproducible and stable contractions, which were relaxed in a concentration-dependent fashion by 5-HT or 5-methoxytryptamine (Table 1). In both cases 5-HT was the more potent agonist. As has previously been shown in carbachol-precontracted tissue (Baxter et al., 1991), 5-HT relaxation of substance P-precontracted tissue was unaffected by tetrodotoxin (Table 2). The substituted benzamides also relaxed precontracted tissue with a similar order of potency in substance P- and carbachol-precontracted tissue of cisapride > (*R*)-zacopride > lintopride > metoclopramide (Table 1). Each of the substituted benzamides acted as a full agonist (α not different to unity) with the exception of cisapride which was a partial agonist in carbachol- but not substance P-precontracted tissue (Table 1).

3.2. Receptor antagonist sensitivity

The concentration–response curve for 5-HT was not significantly altered by the neurotoxin tetrodotoxin, the 5-HT_1/5-HT_2 receptor antagonist methysergide or by the 5-HT_3 receptor antagonist ondansetron (Table 2) in either substance P- or carbachol-precontracted tissue. Although the response to substance P and carbachol was unaffected by the 5-HT_4 receptor antagonist, GR113808, the response to 5-HT was shifted to the right in a competitive fashion yielding a pK_b of 9.51 (9.46–9.57) and 9.81 (9.78–9.91) in substance P- and carbachol-precontracted tissue, respectively (Fig. 1, Table 2). The shift in both substance P- and carbachol-precontracted tissue was parallel as determined using analysis of variance. When compared statistically there was no difference in the sensitivity of substance P- and 5-HT-precontracted tissue to GR113808 ($P > 0.05$).

4. Discussion

The present study confirms earlier reports that 5-HT post-synaptically reduces the contractile response to carbachol (Baxter et al., 1991; Gale et al., 1994). In these and the present study this effect was suggested to be mediated by 5-HT_4 receptors, as the response to 5-HT was mimicked by cisapride > (*R*)-zacopride > lintopride > metoclopramide, and was inhibited by GR113808. Likewise, and in agreement with previous reports (Baxter et al., 1991), of the tryptamines, 5-HT was slightly more potent than 5-methoxytryptamine. The present study extends this finding by showing for the first time that both the tryptamines and the substituted benzamides also relax substance P-precontracted tissue. The rank order potency of these agonists was similar to relaxation of carbachol-precontracted tissue suggesting action at the same receptor site. Relaxation of both carbachol- (Baxter et al., 1991) and substance P-precontracted tissue by 5-HT involved a

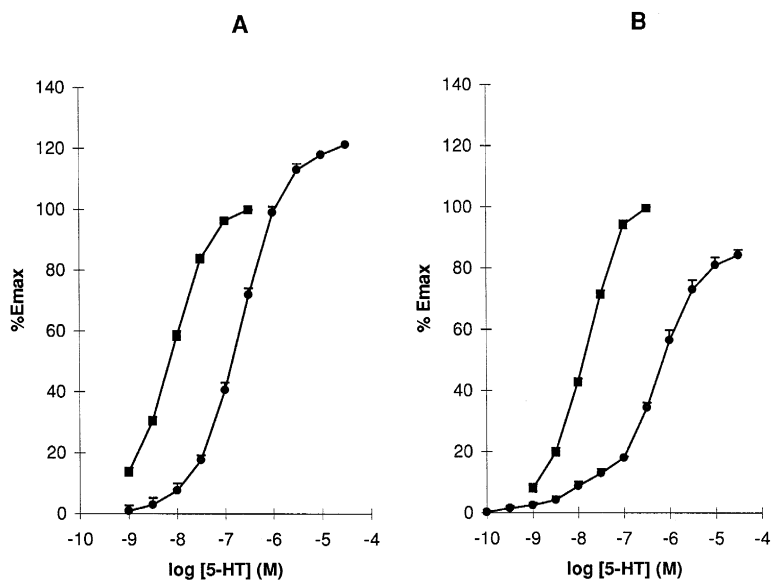

Fig. 1. Inhibition of 5-HT relaxation of (A) substance P- ($n = 3$) and (B) carbachol- ($n = 3$) precontracted oesophageal tunica muscularis mucosae by GR113808 (10 nM). ■ represents control curves and ● represents 5-HT responsiveness in the presence of receptor antagonist.

post-synaptic pathway as it was tetrodotoxin-insensitive. This post-synaptic receptor was confirmed to be of the 5-HT_4 receptor subtype as GR113808 shifted the concentration–response curve to 5-HT to the right with a pK_b similar to that previously described for its 5-HT_4 receptor antagonist properties (Gale et al., 1994). The response to 5-HT was shown to be specific for 5-HT_4 receptors as its concentration–response curve was not affected by methysergide or ondansetron arguing against an involvement of 5-HT_1, 5-HT_2 or 5-HT_3 receptor subtypes.

Since their initial description, 5-HT_4 receptor-mediated effects on intestinal cholinergic nerves have been well documented. In the guinea-pig small and large intestine these effects result from the stimulation of acetylcholine release (Wollard et al., 1994; Kilbinger and Wolf, 1992; Craig and Clarke, 1990). In the human colon, however, only post-synaptic 5-HT_4 receptors have been identified (Tam et al., 1994), and in this respect the rat oesophagus which also contains post-synaptic 5-HT_4 receptors appears to be a particularly good model for the development of receptor antagonists for use in man. This is probably, in part, a reflection of the methodologies used, as nerve cell bodies are present in whole guinea-pig intestinal preparations, but not in isolated human or rat muscle strips. Consequently the rat oesophagus has been frequently used in the screening of new products (see, for example, McLean and Coupar, 1995; Gale et al., 1994). Despite the importance of the post-synaptic 5-HT_4 receptor, it is surprising that its published biological effects have been confined to the modulation of cholinergic excitation. The present data therefore expand our knowledge, demonstrating that a similar 5-HT_4 receptor post-synaptically modulates cholinergic as well as tachykinergic excitation, thus representing functional antagonism. Cyclic AMP-dependent protein kinase has been shown to attenuate phosphoinositide hydrolysis (Zhang and Buxton, 1993), thus providing a mechanism of action for post-synaptic effects of the adenylate cyclase-coupled 5-HT_4 receptor. Both carbachol and substance P have been shown to stimulate inositol trisphoshate production (Zhang and Buxton, 1993; Mallows and Bolton, 1987), and this may provide a common target for the relaxatory effects of 5-HT_4 receptor stimulation in cholinergic and tachykinergic precontracted tissue. If this is the case then 5-HT_4 receptors may be expected to modulate the effect of any excitatory agonist whose mechanism of action involves phosphoinositide hydrolysis.

Thus, in conclusion, the present study shows that post-synaptic modulation of oesophageal smooth muscle is not confined to tissue precontracted with cholinergic agonists but extends to interaction with tachykinergic pathways. This is an especially important observation as the tachykinins have been implicated in a variety of diseases and although one means of therapy may involve specific tachykinergic receptor antagonists, the modulation of substance P-mediated responses through the 5-HT_4 receptor subtype offers a second pharmacological approach.

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