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Contractile responses to substance P and related peptides of the isolated muscularis mucosae of the guinea-pig oesophagus

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1 The site of action of substance P and related tachykinins with respect to isotonic contractions was examined on the isolated muscularis mucosae attached to the submucous plexus of the guinea-pig oesophagus.

2 Substance P (> 30 nM) produced a concentration-dependent contraction of the muscularis mucosae (EC_{50} 1.9 ± 0.5 μM, $n = 10$). The contractions were rapid in onset (2 min or less), sustained, reversible by washing and the preparation did not show tachyphylaxis.

3 Eledoisin and physalaemin produced similar sustained contraction of the muscularis mucosae. The order of sensitivity was eledoisin > substance P > physalaemin. Contractions induced by 1 μM of each tachykinin were not significantly modified by incubation of the tissue with substance P or eledoisin (10 μM for 30 min).

4 The contractile responses to tachykinins were unaffected by tetrodotoxin (0.3 μM), atropine (0.3 μM), phentolamine (1 μM), chlorpheniramine (1 μM), methysergide (1 μM), baclofen (100 μM) and verapamil (10 μM), but were abolished by the incubation of the tissue with calcium-free, EGTA (0.1 mM)-containing Tyrode solution.

5 A substance P antagonist, [D-Pro^2, D-Trp^{7,9}]-substance P (> 0.1 μM), produced a transient contraction of the muscularis mucosae and the smooth muscle regained its original tone within 6 to 10 min. Contractions to the tachykinins were now inhibited by the antagonist (0.1–10 μM) in a concentration-dependent manner, the order of sensitivity being physalaemin > substance P = eledoisin.

6 The cholinergically mediated electrically (0.1 Hz, 0.5 ms, supramaximal voltage)-induced twitch contractions of the muscularis mucosae were not significantly modified by substance P (0.01–0.3 μM).

7 The present results indicate that substance P and related tachykinins contract the isolated muscularis mucosae of the guinea-pig oesophagus by a direct action on the smooth muscle, probably by stimulating SP-E receptors.

Introduction

Since Otsuka and his colleagues presented evidence that substance P might act as a neurotransmitter released by primary sensory neurones in dorsal spinal roots (Otsuka, Konishi & Takahashi, 1975; Otsuka & Konishi, 1976), the peptide has attracted much attention concerning its possible role as a neurotransmitter or neuromodulator in both the central and peripheral nervous systems (Iversen, 1982). By immunohistochemical studies, it has been demonstrated that substance P-containing nerve fibres are found in all layers of the mammalian gut wall (Jessen, Saffrey, van Noorden, Bloom, Polak & Burnstock, 1980; Costa, Furness, Llewellyn-Smith & Cuello, 1981). These enteric neurones are considered to act not only as sensory neurones but also as interneurones or motor neurones, since the peptide produces excitatory responses of the gut by a direct action on smooth muscles and by an indirect action on the intramural nerve plexus (Bury & Mashford, 1977a; Morita, North & Katayama, 1980; Yau & Youther, 1982; Holzer, 1982; Daniel, Gonda, Domoto, Oki & Yanaihara, 1982).

Most physiological and pharmacological studies on the action of substance P in the gastrointestine have

been performed on longitudinal or circular smooth muscle and on the myenteric plexus, which is located between these two muscle layers. However, there is no report based on experiments using the muscularis mucosae and the submucous plexus, which are located in submucosal layers. The isolated muscularis mucosae of the guinea-pig oesophagus contains a submucous plexus which includes cholinergic nerve cell bodies, independent of the myenteric plexus (Kamikawa & Shimo, 1979). This is a suitable preparation for examining the action of drugs on the submucous plexus and muscularis mucosae of the alimentary tract (Kamikawa, Shimo & Uchida, 1982; Kamikawa & Shimo, 1983a,b). Recently, Leander, Brodin, Håkanson, Sundler & Uddman (1982) showed that substance P-immunoreactive nerve fibres are observed in the myenteric and submucous plexuses and the muscularis mucosae of the guinea-pig oesophagus. This led us to examine the action of substance P and related peptides on the submucous plexus and the muscularis mucosae of this tissue.

Methods

Male guinea-pigs (300 to 500 g) were killed by stunning and bleeding, the oesophagus was excised and the isolated muscularis mucosae attached to the submucous plexus was prepared (Kamikawa & Shimo, 1979). Briefly, the excised oesophagus was pinned on a cork mat immersed in Tyrode solution. The outer striated muscle coat was cut longitudinally, and gently peeled away leaving an inner tube. The tube, which included the longitudinal muscularis mucosae and was about 15 mm long (unstretched length), was immersed in a 10 ml organ bath filled with a modified Tyrode solution of the following composition (mM): NaCl 136.8, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 11.9, NaH_2PO_4 0.42, disodium ethylenediaminetetraacetic acid (EDTA) 0.03, ascorbic acid 0.12 and glucose 5.56 (pH 7.4). This solution was bubbled with 5% CO_2 and 95% O_2, and maintained at 37°C.

The preparation was suspended under a 0.3 g load and 60 min was allowed to elapse before experiments were started. During this equilibration period, the tissue was washed with fresh Tyrode solution every 20 min. Responses of the longitudinal muscularis mucosae were recorded by means of an isotonic transducer (MEC-1411) and a Nihon Kohden polygraph (RJG-4004).

In some experiments when the role of external calcium ion in the contractile responses of the muscularis mucosae was investigated, the tissue was incubated with the calcium-free medium, in which $CaCl_2$ was omitted and 0.1 mM ethyleneglycol bis(β-aminoethylether)-tetraacetic acid (EGTA) was added to the Tyrode solution described above. Electrical stimulation of intramural cholinergic nerves in the isolated muscularis mucosae was carried out by means of two coaxial platinum electrodes, the anode in the lumen and the cathode in the organ bath. To obtain stable twitch-like contractions, the stimulus parameters were 0.1 Hz, 0.5 ms and supramaximal voltage (approx. 40 V) (Kamikawa *et al.*, 1982).

The data obtained are expressed as mean ± s.e.mean. Each experimental group consisted of 3–10 preparations taken from different animals. The concentrations of spasmogens required to produce 50% maximal contraction (EC_{50}) was calculated from individual concentration-response curves. Students's t tests for paired or unpaired observations were used for statistical evaluation of the data. P values smaller than 0.05 were considered to be significant.

Drugs used were substance P, eledoisin, physalaemin, [D-Pro^2, D-Trp^{7,9}]-substance P (Protein Research Foundation), tetrodotoxin, chlorpheniramine maleate (Sankyo), atropine sulphate (Wako), acetylcholine chloride (Daiichi), methysergide (Sandoz), baclofen and phentolamine hydrochloride (CIBA-Geigy). All drugs were dissolved in 0.9% w/v NaCl solution (saline). All peptides were diluted in plastic tubes immediately before use. The molar concentrations of drugs described in this paper refer to the final bath concentrations.

Results

Response of muscularis mucosae of guinea-pig oesophagus to tachykinins

The isolated muscularis mucosae of the guinea-pig oesophagus usually showed neither tone nor spontaneous activity. Substance P, above 30 nM, produced a contraction of the muscularis mucosae which had a rapid onset, reaching a peak in about 1–2 min (Figure 2a). When substance P was left in the organ bath, the contraction was sustained at the level of about 60–80% of the peak response for 30 min. Contraction to substance P (2 μM) was fully reversible on washing, and repetitive applications every 10 min produced consistent contractile responses. The response to substance P was concentration-dependent and the EC_{50} was 1.9 ± 0.5 μM ($n = 10$) (Figure 1 and Table 1). Similar contractions of the muscularis mucosae were also obtained with the other tachykinins, eledoisin or physalaemin; their EC_{50} values were 0.3 or 8.3 μM, respectively (Figures 1 and 2, Table 1). However, as shown in Figure 1, the maximum contractions induced by 10 μM of each tachykinin were less than 70% of that induced by acetylcholine (10 μM). Submaximal contractions in-

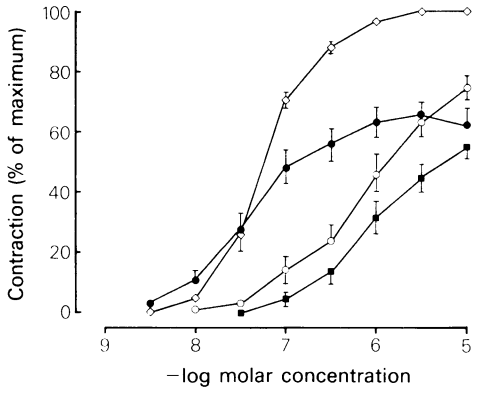

Figure 1 Cumulative concentration-response curves for the contractile responses to acetylcholine (◇, $n = 10$), substance P (○, $n = 10$), eledoisin (●, $n = 8$) and physalaemin (■, $n = 8$) of the isolated muscularis mucosae of the guinea-pig oesophagus. Each point represents the mean response; vertical lines show s.e.mean. Concentrations of tachykinins above 10 μM could not be prepared from the commercial peptides used here.

duced by 1 μM of each tachykinin were not modified by 30 min incubation of the muscularis mucosae with substance P (10 μM) or eledoisin (10 μM) ($n = 4$).

Effect of antagonists

The contractile responses to substance P, eledoisin and physalaemin were unaffected by tetrodotoxin (0.3 μM, $n = 5$), atropine (0.3 μM, $n = 8$), phentolamine (1 μM, $n = 3$), chlorpheniramine (1 μM, $n = 8$), methysergide (1 μM, $n = 4$) or baclofen (100 μM, $n = 6$). Verapamil (10 μM, $n = 12$) did not modify the amplitude of contractions to three tachykinins (2 μM), but abolished the weak rhythmical contractions superimposed on the contracture (Figure 2). However, the contractile responses to the

Table 1 Concentrations causing 50% maximal contraction (EC_{50}) for tachykinin, [D-Pro^2, D-Trp^{7,9}]-substance P and acetylcholine in the isolated muscularis mucosae of the guinea-pig oesophagus

Agonist	n	*EC_{50} (μM)*	*Relative potency*
Substance P	10	1.9 ±0.5	1.0
Physalaemin	8	8.3 ±2.7	0.2
Eledoisin	8	0.3 ±0.12	6.3
[D-Pro^2, D-Trp^{7,9}]-substance P	10	0.28±0.06	6.8
Acetylcholine	10	0.06±0.006	31.7

Relative potency = EC_{50} of substance P/EC_{50} of each agonist. EC_{50} values are mean ± s.e.mean.

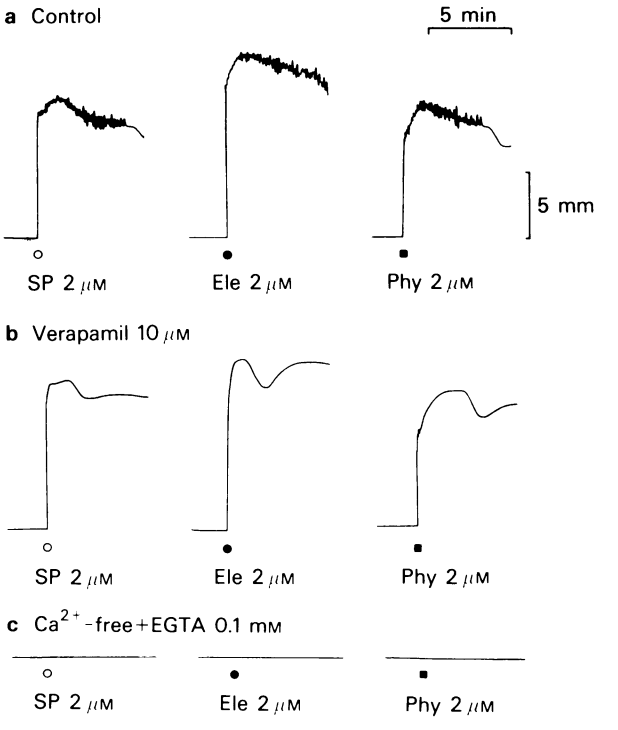

Figure 2 Contractions of the isolated muscularis mucosae of the guinea-pig oesophagus induced by substance P (SP, 2 μM, ○), eledoisin (Ele, 2 μM, ●) and physalaemin (Phy, 2 μM, ■). (a) Control responses; (b) responses in the presence of verapamil (10 μM); (c) responses in the calcium-free, EGTA 0.1 mM containing Tyrode solution. The responses to the three tachykinins were not inhibited by verapamil, but were abolished by incubation of the tissue with calcium-free medium. Vertical calibration shows 5 mm shortening of the tissue; horizontal calibration is 5 min. The results shown in (a), (b) and (c) are obtained from the same preparation.

three tachykinins could not be obtained in preparations suspended in calcium-free, EGTA (0.1 mM)-containing Tyrode solution.

A putative substance P antagonist, [D-Pro^2, D-Trp^{7,9}]-substance P (> 0.1 μM), contracted the isolated muscularis mucosae. These contractions were rapid in onset and reached a peak within 2 min (Figures 3 and 4). When the antagonist was left in the organ bath, the contraction faded and the basal tone was re-established within 6 to 10 min. When the same concentration of [D-Pro^2, D-Trp^{7,9}]-substance P was re-applied to the bath within 20 min after washing out the previous dose, no contraction occurred. This indicates a rapid development of tachyphylaxis. When the basal tone had been re-established without washing out [D-Pro^2, D-Trp^{7,9}]-substance P (3 μM) from the bath, further addition of substance P (2 μM)

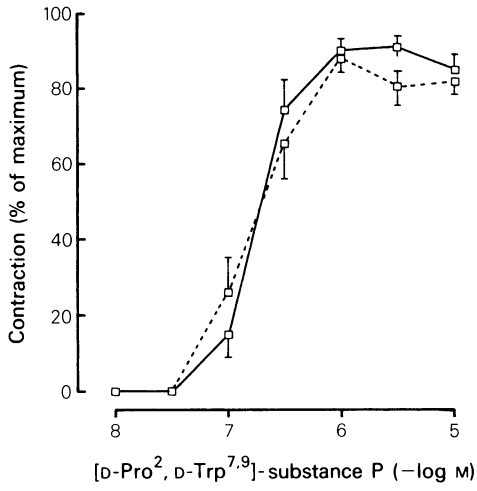

Figure 3 Log concentration-response curves for the contractions of the isolated muscularis mucosae of the guinea-pig oesophagus induced by [D-Pro^2, D-Trp^{7,9}]-substance P in the absence (solid line, $n = 10$) or presence (dotted line, $n = 10$) of chlorpheniramine (1 μM). Each concentration of [D-Pro^2, D-Trp^{7,9}]-substance P was applied to the tissue separately with a 30 min interval between doses. Ordinate scale, % maximal contraction induced by acetylcholine (3 μM). Each point represents the mean response; vertical lines show s.e.mean.

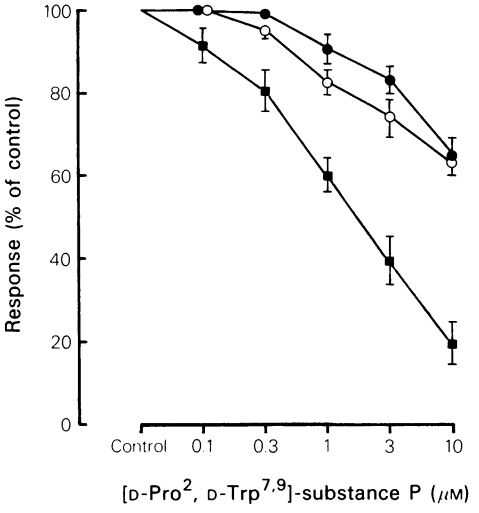

Figure 5 A comparison of the antagonism by [D-Pro^2, D-Trp^{7,9}]-substance P of the contractions induced by substance P (2 μM, ○, $n = 9$), eledoisin (2 μM, ●, $n = 8$) and physalaemin (2 μM, ■, $n = 8$) in the isolated muscularis mucosae of the guinea-pig oesophagus. Each tachykinin was applied to the tissue when the tone induced by the antagonist had declined to the basal level without washing out. The ordinate scale shows the amplitude of the responses as a percentage of the control. Each point represents the mean response; vertical lines show s.e.mean.

produced a contraction which was slightly reduced in amplitude (Figure 4). The inhibitory action of the antagonist was reversible, and 30 min after washing out, the response to substance P was restored to the control level. As shown in Figure 5, [D-Pro^2, D-Trp^{7,9}]-substance P (> 0.1 μM) inhibited contractions to 2 μM of each tachykinin, in a concentration-dependent manner, being most effective against physalaemin, but less effective against substance P and eledoisin.

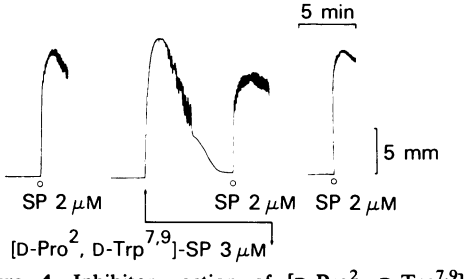

Figure 4 Inhibitory action of [D-Pro^2, D-Trp^{7,9}]-substance P (3 μM) on the substance P (SP, 2 μM)-induced contraction of the isolated muscularis mucosae of the guinea-pig oesophagus. When the tone induced by [D-Pro^2, D-Trp^{7,9}]-substance P had declined to the basal level without washing out the peptide, further addition of substance P produced a contracture which has slightly reduced in magnitude. After 30 min washing out of the antagonist, contraction to substance P was restored to the control response. Vertical calibration shows 5 mm shortening of the tissue; horizontal calibration is 5 min.

Effect of electrical stimulation

Transmural electrical stimulation (0.1 Hz, 0.5 ms, supramaximal voltage) of the isolated muscularis mucosae of the guinea-pig oesophagus produced stable twitch-like contractions, which were mediated by stimulation of intramural cholinergic nerves in this tissue (Kamikawa & Shimo, 1979; Kamikawa *et al.*, 1982). Substance P (up to 0.1 μM) had no significant effect on the electrically-induced twitch (Figure 6) ($n = 6$). Substance P (> 0.1 μM) increased the basal tone of the preparation and, therefore, its effects on the twitches could not be quantified precisely.

Discussion

These experiments demonstrate that substance P contracts the muscularis mucosae of the guinea-pig oesophagus by a direct action on the smooth muscle and excludes an indirect effect on the attached submucous plexus, or mast cells. The contractions were

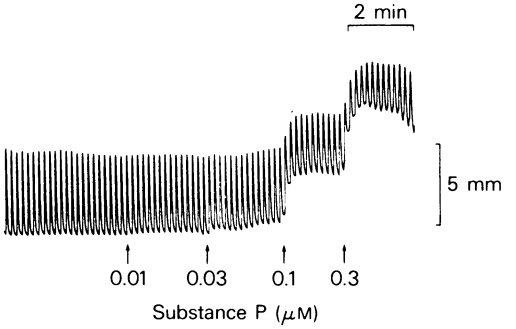

Figure 6 The effect of cumulatively applied substance P on the twitch contractions of the isolated muscularis mucosae of the guinea-pig oesophagus induced by electrical stimulation (0.1 Hz, 0.5 ms, supramaximal voltage). Vertical calibration shows 5 mm shortening of the tissue; horizontal calibration shows 2 min.

unaffected by tetrodotoxin, atropine and chlorpheniramine, and the cholinergically mediated electrically-induced twitch responses of this tissue were also unaffected by substance P. A similar direct action on the smooth muscle occurred with eledoisin, a molluscan tachykinin, and physalaemin, an amphibian tachykinin. The order of potency was eledoisin > substance P > physalaemin.

The contractile responses to tachykinins of the guinea-pig oesophageal muscularis mucosae had the following characteristics: (1) The pattern of contractures was a sustained increase in tone which was overlapped with weak rhythmic motility. (2) Tachyphylaxis by repetitive applications of, or desensitization by long-term application of, tachykinins was not observed. (3) The responses were resistant to verapamil but were completely abolished by omitting the extracellular calcium ion. These characteristics contrast with those found previously in other gastrointestinal smooth muscle preparations. In the guinea-pig ileum, the contractile responses to tachykinins had the following characteristics: (1) The pattern of contractures consisted of phasic and tonic components, and the latter soon began to fade. (2) A rapid development of tachyphylaxis or desensitization was observed to the action of tachykinins. (3) The order of potency was physalaemin > substance P > eledoisin. (4) Substance P stimulated intramural cholinergic nerves in the myenteric plexus (Hedqvist & von Euler, 1975; Bury & Mashford, 1977a, b; Erspamer, Erspamer & Piccinelli, 1980; Holzer, Emson, Iversen & Sharman, 1981; Holzer, 1982; Huidobro-Toro, Chelala, Bahouth, Nodar & Musacchio, 1982; Lee, Iversen, Hanley & Sandberg, 1982; Yau & Youther, 1982).

The different characteristics of the contractile responses to tachykinins of these two preparations can be explained by the concept of multiple tachykinin receptors; the existence of heterogeneous populations of the tachykinin receptor has been postulated in peripheral tissues (Erspamer *et al.*, 1980; Lee *et al.*, 1982; Watson, Sandberg, Hanley & Iversen, 1983). The longitudinal muscles of the guinea-pig ileum contain SP-P type receptors, where physalaemin acts as the most potent agonist, while the rat vas deferens contains SP-E type receptors, where eledoisin acts as the most potent agonist. Therefore, the order of potency of tachykinins observed in the present experiments suggests that the guinea-pig oesophageal muscularis mucosae contains the SP-E type tachykinin receptors. This may also be supported by the antagonistic potency of newly synthesized substance P analogues such as [D-Pro^2, D-Trp^{7,9}]- or [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-substance P. These substance P analogues antagonized the contractile responses to tachykinins of the guinea-pig ileum (Björkroth, Rosell, Xu & Folkers, 1982) and taenia coli (Leander, Håkanson, Rosell, Folkers, Sundler & Tornqvist, 1981; Håkanson, Hörig & Leander, 1982), all of which have SP-P type receptors, in a competitive manner, but failed to inhibit the response to substance P of the hamster urinary bladder, which has SP-E type receptors (Rosell, Björkroth, Xu & Folkers, 1983). In the present tissue, [D-Pro^2, D-Trp^{7,9}]-substance P showed only a weak inhibition on the substance P-induced contractions. In addition, the substance P analogue produced a transient contraction of the muscularis mucosae, which is unlikely to be mediated by endogenous histamine release from mast cells, as reported in the guinea-pig taenia coli (Håkanson *et al.*, 1982), since a histamine H_1-antagonist, chlorpheniramine, failed to inhibit the response. Spasmogenic actions of substance P analogues with antagonistic potency have also been observed in the guinea-pig or rat urinary bladder (Leander *et al.*, 1981; Rosell *et al.*, 1983) and in the sphincter pupillae muscle of the rabbit eye (Mandahl & Bill, 1983).

The failure of the calcium antagonist, verapamil, to inhibit the contractile responses to tachykinins on the guinea-pig oesophageal muscularis mucosae also contrasts with the results in the guinea-pig ileum, where verapamil or D-600 effectively inhibited the substance P-induced contracture (Bury & Mashford, 1976; Milenov, Nieber & Oehme, 1978). Since contractions to tachykinins were completely abolished by omitting the extracellular calcium ion in both preparations, responses to tachykinins require an influx of extracellular calcium ions in both the oesophageal muscularis mucosae and the ileal smooth muscle. There are at least two types of calcium ion channels that could be involved with the contractions, potential-dependent and receptor-operated ones (Bolton, 1979). Calcium antagonists, such as verapamil, selectively block the potential-

dependent calcium ion channels, but not the receptor-operated one. The present findings suggest that, in the oesophageal muscularis mucosae, tachykinins open the calcium ion channels independently of the membrane potential. In the guinea-pig ileum, however, substance P inactivates potassium conductance and causes membrane depolarization which can open the potential-dependent calcium ion channel (Fujisawa & Ito, 1982; Holzer & Petsche, 1982).

In conclusion, substance P and related tachykinins produce a contraction of the isolated muscularis mucosae of the guinea-pig oesophagus, which is probably mediated by different types of the tachykinin receptor (SP-E receptor) and different mechanisms of the calcium entry from those in the ileal longitudinal muscles. Therefore, the present tissue is a useful *in vitro* preparation to study the mechanism of action of tachykinins.

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