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Neurokinin$_3$-receptors are linked to inositol phospholipid hydrolysis in the guinea-pig ileum longitudinal muscle-myenteric plexus preparation

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1 Tachykinin-stimulated inositol phospholipid hydrolysis was examined in slices of longitudinal muscle from guinea-pig ileum.

2 Substance P, neurokinin A and neurokinin B induced a concentration-dependent accumulation of total [^{3}H]-inositol phosphates in the presence of 12 mM lithium with similar maximal responses and EC_{50} values.

3 The selective NK_1-receptor agonist, substance P methyl ester, and the selective NK_3-receptor agonist succ-[Asp^6, MePhe^8]-SP(6–11) (senktide) also stimulated [^{3}H]-inositol phosphate formation with maximum responses of 50.69 ± 0.96 and 45.64 ± 1.17% relative to 10 μM substance P, respectively. Substance P methyl ester was approximately equipotent with substance P, whereas senktide was approximately 100 times more potent.

4 When added together, maximally effective concentrations of substance P methyl ester and senktide gave responses that were fully additive. In contrast, responses to substance P and neurokinin B were not additive.

5 The stimulation of [^{3}H]-inositol phosphate formation by substance P, neurokinin B and senktide was not affected by atropine (2 μM) or tetrodotoxin (TTX, 0.3 μM).

6 The contractile effect of senktide was inhibited completely by TTX and partially blocked by atropine. Contractions induced by substance P methyl ester were not changed in the presence of TTX or atropine.

7 [D-Pro^4, D-Trp^{7,9,10}]-SP(4-11) competitively antagonized the action of substance P methyl ester on inositol phospholipid hydrolysis and contraction, but had no significant effect on senktide-induced inositol phospholipid breakdown or contraction.

8 These results suggest that NK_3-receptors in the guinea-pig ileum are coupled to inositol phospholipid hydrolysis.

Introduction

There is a great deal of evidence suggesting the existence of multiple receptors for substance P and the other mammalian tachykinins neurokinin A and neurokinin B (see reviews by Watson, 1984a; 1987; Regoli *et al.*, 1987). The original subdivision of tachykinin receptors into 'SP-P' (NK_1) and 'SP-E' (NK_2-receptors), based on rank order potencies of tachykinins in guinea-pig ileum and rat vas deferens respectively (Lee *et al.*, 1982), has more recently been extended to include a third tachykinin receptor subtype, the 'SP-N' (NK_3) receptor, identified on myenteric plexus neurones of the guinea-pig ileum (Laufer *et al.*, 1985), the rat portal vein (McKnight & Maguire, 1987) and in the mammalian central nervous system (Lee *et al.*, 1986; Bergström *et al.*, 1987) (receptor nomenclature as agreed by the participants in the symposium, 'Substance P and Neurokinins – Montreal '86'; see Henry *et al.*, 1987).

At the NK_1-receptor, substance P and the other mammalian tachykinins are approximately equipotent (Osakada *et al.*, 1986). On the other hand, at the NK_2-subtype, neurokinin A and neurokinin B are

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active at nanomolar concentrations and are approximately two orders of magnitude more potent than substance P. In addition, the existence of NK_1- and NK_2-receptors has been reinforced by the development of a selective NK_1-receptor agonist, substance P methyl ester, which, although approximately equipotent with substance P on NK_1 systems, is approximately 100 times less potent than substance P on NK_2 systems (Watson *et al.*, 1983). The recently described NK_3-receptor exhibits a pharmacological profile distinct from either the NK_1- or NK_2-receptor subtype. Here, neurokinin A and substance P are approximately two orders of magnitude less potent than neurokinin B (Wormser *et al.*, 1986). The existence of NK_3-receptors has been confirmed by the recent availability of a highly potent and selective NK_3-receptor agonist, succinyl-[Asp^6, MePhe^8]-SP(6-11) (senktide), which is approximately 60,000 times more selective for NK_3- over NK_1- or NK_2-receptors (Wormser *et al.*, 1986).

Substance P and related tachykinins have been shown to stimulate inositol phospholipid hydrolysis in a variety of tissues, including rat parotid gland (Hanley *et al.*, 1980), guinea-pig ileum longitudinal muscle and rat hypothalamus (Watson & Downes, 1983), examples of NK_1-type tissues. This biochemical response has also been observed in NK_2-type tissues such as the rat ileum (Watson, 1984b) and hamster urinary bladder (Bristow *et al.*, 1987). We have therefore investigated the possibility that the NK_3-receptor may also be coupled to inositol phospholipid hydrolysis. A preliminary account of this work has been presented to the British Pharmacological Society (Guard *et al.*, 1987).

Methods

Agonist-induced accumulation of [^{3}H]-inositol phosphates

Hartley strain guinea-pigs of either sex (300–500 g) were killed by cervical dislocation. The entire length of the small intestine, apart from the duodenum, was removed into pre-gassed (95% O_2 : 5% CO_2) Krebs solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 10 (pH 7.4) at room temperature. Cross-chopped slices (350 × 350 μm) of ileal smooth muscle (from longitudinal muscle strips of guinea-pig small intestine, prepared essentially as described by Rang, 1964) were prepared with a McIlwain tissue chopper. The slices were washed twice with 10 ml Krebs solution and incubated in 5 ml Krebs containing 30 μCi [^{3}H]-*myo*-inositol under an atmosphere of 95% O_2 : 5% CO_2 for 1 h in a shaking water bath at 37°C. The prelabelled slices were subsequently washed with 10 ml Krebs solution at 37°C every 15 min for 1 h.

Ileal slices were suspended in 2 ml of Krebs and 40 μl aliquots were transferred to plastic tubes containing 200 μl of pre-gassed Krebs to which 12 mM LiCl had been added. Agonists were then added in 10 μl volumes, the tubes gassed, capped and incubated in a shaking water bath at 37°C for 30 min. In experiments where atropine, tetrodotoxin or tachykinin antagonist were used, tissue was incubated with these drugs 10 min before exposure to agonists.

Extraction of water-soluble [^{3}H]-inositol phosphates

Incubations were terminated by the addition of 0.94 ml chloroform : methanol : HCl (100 : 200 : 1, v/v/v). Total [^{3}H]-inositol phosphates were extracted essentially as described by Berridge *et al.* (1982). Chloroform (0.31 ml) and water (0.31 ml) were added, the samples vortexed and the phases separated by centrifugation at 1000 *g* for 5 min. A portion of the upper aqueous phase (0.9 ml) was applied to a glass column containing 1 ml of an approximately 1 : 1 (w/v) slurry of Dowex AG 1-X8 resin (100–200 mesh, formate form) and distilled water. [^{3}H]-inositol and [^{3}H]-glycerophosphoinositol were removed with 5 mM disodium tetraborate/60 mM ammonium formate (2 × 8 ml). Total [^{3}H]-inositol phosphates (inositol mono-, bis- and tris-phosphates) were eluted with 6 ml of 800 mM ammonium formate/0.1 M formic acid. This eluant was collected in scintillation vials and counted in the gel phase for radioactivity following the addition of 10 ml Liquiscint. When slices were stimulated for 30 min with 1 μM substance P and the individual [^{3}H]-inositol phosphates separated, approximately 90% of the radioactivity was recovered in the inositol monophosphate peak. Individual [^{3}H]-inositol phosphates were eluted in 10 × 1 ml fractions of (1) 200 mM ammonium formate/0.1 M formic acid (for inositol monophosphate); (2) 400 mM ammonium formate/0.1 M formic acid (for inositol bisphosphate); (3) 800 mM ammonium formate/0.1 M formic acid (for inositol trisphosphate) and 1.2 M ammonium formate/0.1 M formic acid (for inositol tetrakisphosphate). There was no detectable increase in inositol tetrakisphosphate levels following stimulation of slices for 30 min with 1 μM substance P and therefore 800 mM ammonium formate/0.1 M formic acid was used to elute 'total', i.e., inositol mono-, bis-, and trisphosphates.

Organ bath measurements

Longitudinal muscle strips were suspended in 3 ml of Krebs solution gassed with O_2/CO_2 (95 : 5) at 37°C in a silanised organ bath and allowed to equilibrate

for 30–60 min with washing at every 15 min. Serial concentration-response curves were constructed with agonists added at 4 min intervals, and left in contact with the tissue for 20–30 s. Antagonists were given for an initial 10 min period and concentration-response curves were determined as described above with antagonists being added 3 min prior to agonist. Contractions were recorded isotonically under a resting tension of 1.5 g.

Analysis of results

Concentration-response curves were constructed for tachykinin-induced accumulation of total [^{3}H]-inositol phosphates by combining data from three or more experiments. Within an experiment, all data points were determined in triplicate or quadruplicate. The response to 1 mM carbachol was measured in all experiments and was used to correct for differences in the absolute level of accumulated [^{3}H]-inositol phosphates ([^{3}H]-IP) between slice preparations. The concentration-response curves were analysed using the program FIT (Barlow, 1983), where:

Stimulation of [^{3}H]-IP accumulation

$$= E_{max} \times D^n/(D^n + (EC_{50})^n)$$

D is the agonist concentration, n is the Hill coefficient, EC_{50} is the concentration of agonist giving half maximal stimulation and E_{max} is the maximal stimulation.

The affinity constant of the tachykinin antagonist against substance P methyl ester-induced [^{3}H]-IP accumulation was obtained from parallel shifts of the concentration-response curves to substance P methyl ester using the relationship:

$$\text{Concentration-ratio} = [A] \times K_a + 1$$

where [A] is the concentration of antagonist and K_a the affinity constant. The concentration-ratio is the concentration of substance P methyl ester required for a given response in the presence of antagonist divided by the concentration required in the absence of antagonist. Statistical comparisons were made by Student's *t* test.

Chemicals

Myo-[2-^{3}H]-inositol (17.9 Ci mmol^{-1}) was purchased from Amersham International plc and purified before use by passing through a column of Dowex-1 resin (formate form). Atropine sulphate and tetrodotoxin were obtained from Sigma, Poole, Dorset, and histamine acid phosphate and carbachol from BDH, Atherstone, Warwickshire. Dowex AG 1-X8 (100–200 mesh, formate form) was obtained from Bio-rad Laboratories, Richmond, California.

Substance P and neurokinin A were purchased from Peninsula Laboratories, California; substance P methyl ester, neurokinin B and [D-Pro^4, D-Trp^{7,9,10}]-SP(4-11) from Bachem Biochemicals, Basel, Switzerland. The batch of antagonist used in this study has been shown to be 50.5% of total amount of peptide expected (Brown *et al.*, 1986), determined by amino acid analysis and FAB mass spectrometry. Succinyl-[Asp^6, MePhe^8]-SP(6-11) was synthesized and characterized in the Medicinal Chemistry Department of the MSDRL Neuroscience Research Centre. All peptides were dissolved in 0.1% acetic acid and stored in aliquots at −20°C with the exception of neurokinin B and [D-Pro^4, D-Trp^{7,9,10}]-SP(4-11) which were dissolved in dimethylsulphoxide (DMSO).

Results

Tachykinin-stimulated accumulation of total [^{3}H]-inositol phosphates

In the absence of agonist, basal total [^{3}H]-inositol phosphate accumulation was 504 ± 17 d.p.m. per 40 μl slices (n = 28). Following a 30 min incubation in the presence of 1 mM carbachol total [^{3}H]-inositol phosphate production was increased approximately 9 fold to 4591 ± 226 d.p.m. per 40 μl slices (n = 19). Substance P, neurokinin A and neurokinin B produced a concentration-dependent accumulation of total [^{3}H]-inositol phosphates with similar maximum responses and EC_{50} values of 87 ± 15, 84 ± 7 and 28 ± 4 nM respectively (means ± s.e. mean, n = 3). The selective NK_1 agonist, substance P methyl ester, and the selective NK_3 agonist, senktide, also stimulated inositol phospholipid hydrolysis with substance P methyl ester being approximately equipotent with substance P (EC_{50}, 50.0 ± 4.6 nM) and senktide approximately two orders of magnitude more potent than the other tachykinins tested (EC_{50}, 0.54 ± 0.06 nM, Figure 1).

The maximal stimulation of [^{3}H]-inositol phosphate accumulation produced by substance P methyl ester and senktide was 50.69 ± 0.96 and 45.64 ± 1.17% of the response to 10 μM substance P respectively (n = 7). When added together, maximally effective concentrations of substance P methyl ester (10 μM) and senktide (1 μM) were fully additive producing 93.0 ± 4.4% of the response to 10 μM substance P (Figure 2). In contrast, substance P and neurokinin B were not additive (Figure 3). Full additivity was also observed between substance P (10 μM) and a maximally effective concentration of histamine (1 μM, Figure 3).

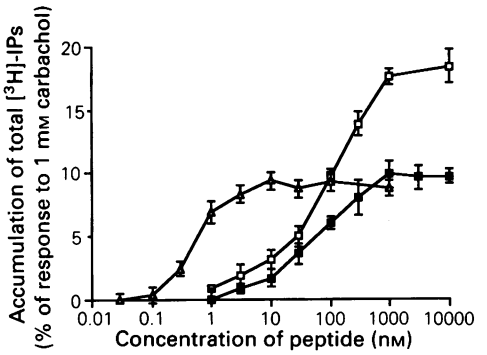

Figure 1 Stimulation of total [^{3}H]-inositol phosphate ([^{3}H]-IP) accumulation in slices of ileal smooth muscle by (□) substance P, (■) substance P methyl ester and (△) senktide. Responses are expressed as a percentage of that produced by 1 mM carbachol, which was measured in all experiments. Each point represents the mean of triplicate or quadruplicate determinations from 3 (substance P) or 7 (substance P methyl ester and senktide) separate experiments; s.e. mean shown by vertical lines.

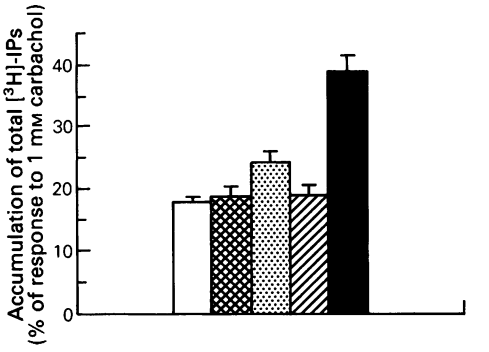

Figure 3 Accumulation of total [^{3}H]-inositol phosphates ([^{3}H]-IPs) in response to maximally-stimulating concentrations of substance P (10 μM, open column), neurokinin B (10 μM, cross-hatched column), histamine (1 mM, stippled column), substance P and neurokinin B added together (hatched column), and substance P and histamine added together (solid column). Results are expressed as the means of triplicate or quadruplicate determinations from 3–4 separate experiments with s.e. mean shown by vertical lines.

Effect of atropine and tetrodotoxin

The accumulation of [^{3}H]-inositol phosphates induced by senktide, substance P and neurokinin B was both atropine and tetrodotoxin-insensitive (Table 1). In contrast, the senktide-induced contraction of longitudinal muscle strips was partially blocked by atropine (2 μM) with the concentration-response curve being shifted to the right in a non-parallel manner and the maximum reduced by approximately 60%. Tetrodotoxin (0.3 μM) completely inhibited the contractile response to senktide (Figure 4). The concentration-response curve to substance P methyl ester for contraction was not

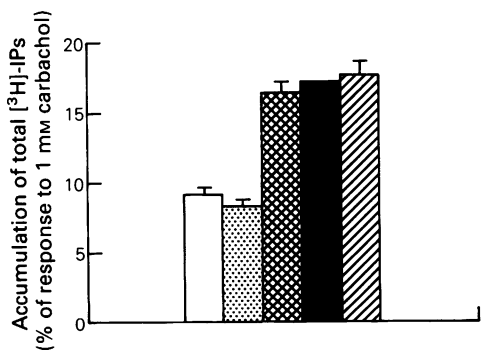

Figure 2 Accumulation of total [^{3}H]-IPs (inositol phosphates) in response to maximally stimulating concentrations of senktide (1 μM, open column), substance P methyl ester (10 μM, stippled column), senktide and substance P methyl ester added together (cross-hatched column), and substance P (10 μM, hatched column). Results are expressed as the means of triplicate or quadruplicate determinations from 7 separate experiments with s.e. mean shown by vertical lines. The solid colum, (predicted column) represents the value obtained from combining the mean responses to substance P methyl ester (stippled column) and senktide (open column).

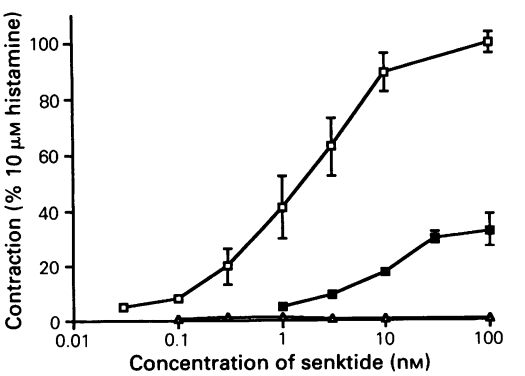

Figure 4 Inhibition by atropine and tetrodotoxin of senktide-induced contraction of ileal longitudinal muscle strips. Concentration-response curves for senktide were obtained in the absence (□) and presence of 2 μM atropine (■) or 0.3 μM tetrodotoxin (△). Responses are expressed relative to a maximal dose of histamine. Each point represents the mean of 3–4 separate experiments with s.e. mean shown by vertical lines.

Table 1 Effect of atropine and tetrodotoxin on tachykinin-induced [^{3}H]-inositol phosphate accumulation in longitudinal muscle slices of the guinea-pig ileum

Drugs	*Concentration* (μM)	n	% *total* [^{3}H]-*IP formation* *Control*	*+ Atropine*	*+ TTX*
Basal	—	3	100 ± 4.2	112 ± 11	93 ± 8
Substance P	10	4	257 ± 15	263 ± 15	265 ± 20
Neurokinin B	10	3	280 ± 20	287 ± 26	291 ± 23
Senktide	1	3	190 ± 11	188 ± 11	186 ± 9
Carbachol	1000	3	929 ± 68	133 ± 6*	n.d.

Results are expressed relative to basal (100% = no effect), and are expressed as the mean ± s.e. mean. Incubation time with agonist was 30 min. Atropine (2 μM) or TTX (tetrodotoxin, 0.3 μM) were added 10 min prior to addition of agonist; n = number of independent experiments; IP = inositol phosphate.
* $P < 0.001$, n.d. = not determined.

affected by atropine or tetrodotoxin (data not shown).

Effect of [D-*Pro*^{4}, D-*Trp*^{7,9,10}]-*SP(4-11)*

The tachykinin antagonist [D-Pro^4, D-Trp^{7,9,10}]-SP(4-11), (15 μM), which alone did not alter basal [^{3}H]-inositol phosphate levels (108 ± 3% of control, $n = 6$), produced a rightward parallel shift of the concentration-response curve to substance P methyl ester ($K_a = 5.07 \times 10^5\ M^{-1}$; $n = 4$), but had no significant effect on [^{3}H]-inositol phosphate accumulation induced by senktide (Figures 5a and b). Similar results were also observed on contraction where this antagonist produced a parallel shift in the substance P methyl ester concentration-response curve with an affinity constant of $7.32 \pm 1.42 \times 10^5\ M^{-1}$ ($n = 3$; Figure 6) but had no effect on the response to senktide (data not shown).

[D-Pro^4, D-Trp^{7,9,10}]-SP(4-11) had no significant effect on the accumulation of [^{3}H]-inositol phosphates following a 30 min incubation in the presence of an almost maximal dose of histamine (0.1 mM, Donaldson & Hill, 1985). Incubation with 0.1 mM histamine produced a 21.52 ± 0.82% increase in total [^{3}H]-inositol phosphate formation (expressed as a percentage of the maximal response to carbachol). In the presence of 15 μM [D-Pro^4, D-Trp^{7,9,10}]-SP(4-11) the increase in [^{3}H]-inositol phosphates by 0.1 mM histamine was 20.12 ± 1.20% ($n = 4$). Hence, we find no evidence in support of the suggestion of Bailey *et al.* (1987) that this antagonist may inhibit inositol phosphate formation non-selectively.

Discussion

The results presented in this study strongly support the concept that NK_3-receptors in the longitudinal muscle-myenteric plexus preparation of the guinea-pig ileum are coupled to inositol phospholipid hydrolysis. The selective NK_1-agonist, substance P methyl ester and the selective NK_3-agonist, senktide,

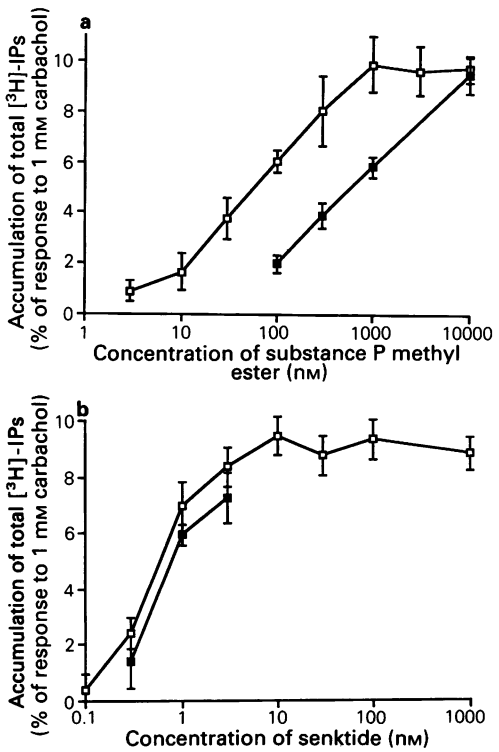

Figure 5 Effect of [D-Pro^4, D-Trp^{7,9,10}]-SP(4-11) on (a) substance P methyl ester and (b) senktide-induced accumulation of total [^{3}H]-inositol phosphates ([^{3}H]-IPs). Each point represents the mean of triplicate or quadruplicate determinations in the absence (□) or presence (■) of 15 μM antagonist from 4 separate experiments; s.e. mean shown by vertical lines.

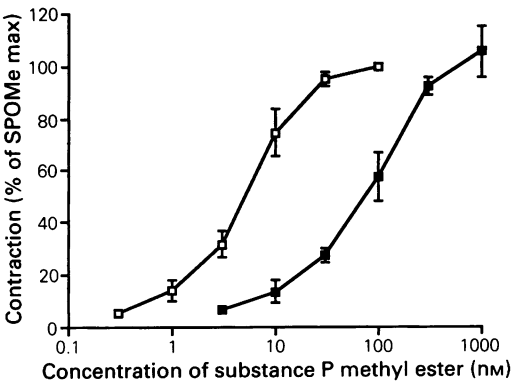

Figure 6 Inhibition by [D-Pro^4, D-Trp^{7,9,10}]-SP(4-11) of substance P methyl ester (SPOMe)-induced contraction of ileal longitudinal strips. Concentration-response curves were obtained in the absence (□) and presence (■) of 15 μM [D-Pro^4, D-Trp^{7,9,10}]-SP(4-11). Each point represents the mean of 3 separate experiments with s.e. mean shown by vertical lines.

were fully additive on inositol phosphate formation producing an increase in [^{3}H]-inositol phosphates which was not significantly different from the maximum response to substance P, suggesting the existence of two distinct populations of tachykinin receptors coupled to inositol phospholipid hydrolysis in this preparation. The selective antagonism of substance P methyl ester, but not senktide-induced [^{3}H]-inositol phosphate accumulation by [D-Pro^4, D-Trp^{7,9,10}]-SP(4-11) confirms this hypothesis. The affinity constant obtained for the inhibition of substance P methyl ester-induced accumulation of [^{3}H]-inositol phosphates by the tachykinin antagonist is in agreement with that obtained for the inhibition of substance P methyl ester-induced contraction of longitudinal muscle strips. Furthermore, [D-Pro^4, D-Trp^{7,9,10}]-SP(4-11) had no significant effect on contraction induced by senktide.

Various tachykinins are capable of stimulating [^{3}H]-acetylcholine release from previously labelled stores within the myenteric plexus of the guinea-pig ileum (Holzer & Lembeck, 1980; Yau & Youther, 1982; Fosbraey *et al.*, 1984). It is possible, therefore, that increases in [^{3}H]-inositol phosphates observed in response to tachykinins may be mediated, in part, via acetylcholine released from myenteric plexus neurones. However, from the experiments performed in the presence of atropine and tetrodotoxin it would appear that the accumulation of [^{3}H]-inositol phosphates in response to tachykinins in this preparation is due to the activation of specific tachykinin receptors rather than indirectly via the release of acetylcholine or some other neurotransmitter from enteric neurones. Given the finding that the inositol phospholipid response to tachykinins is both atropine- and tetrodotoxin-insensitive, it would appear unlikely that sufficient acetylcholine is released to produce a detectable increase in [^{3}H]-inositol phosphate formation. A much higher muscarinic receptor reserve for carbachol-induced contraction of the guinea-pig ileum compared to inositol phospholipid hydrolysis is consistent with the difference in the EC_{50} values for carbachol-induced contraction (39 nM; Burgen & Spero, 1968) and inositol phosphate formation (36 μM; Freedman, 1986).

The contractile response to senktide is partially atropine-sensitive but is fully inhibited by tetrodotoxin. This result suggests that NK_3-receptors are located on enteric neurones in this preparation and that the contraction is mediated via the release of acetylcholine and another transmitter, probably substance P and/or neurokinin A (Franco *et al.*, 1979). The hypothesis that tachykinin-evoked acetylcholine release in the guinea-pig ileum is mediated by NK_3-receptors on the enteric nerves is strongly supported by the inability of [D-Pro^4, D-Trp^{7,9,10}]-SP(4-11) to block tachykinin-evoked acetylcholine release (Featherstone *et al.*, 1986) and senktide-induced inositol phosphate formation and contraction (this study).

In conclusion, these results provide evidence for NK_3-receptor-mediated inositol phospholipid hydrolysis in the guinea-pig ileum longitudinal muscle-myenteric plexus preparation. Hence, it would appear that all three tachykinin receptor subtypes (NK_1, NK_2 and NK_3) are coupled to the same second messenger system.

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