Actions of tachykinins on the rabbit mesenteric artery: substance P and [Glp⁶,L-Pro⁹]SP_{6–11} are potent agonists for endothelial neurokinin-1 receptors

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- 1 Identification of the subtype of neurokinin receptor on the endothelium of the rabbit mesenteric artery was demonstrated by comparing the relative potencies of the naturally occurring tachykinins, substance P, neurokinin A and neurokinin B and the highly selective agonists [Glp⁶,L-Pro⁹]SP_{6–11} (L-Pro), [Glp⁶,D-Pro⁹]SP_{6–11} (D-Pro) and N-succinyl-[Asp⁶,MePhe⁸]SP_{6–11} (senktide).
- 2 Relaxations of the rabbit mesenteric artery to substance P, neurokinin A and neurokinin B were concentration-dependent and were abolished by the removal of the endothelium.
- 3 Substance P was more potent than neurokinin A or neurokinin B and L-Pro was more potent than D-Pro or senktide.
- 4 Substance P, neurokinin A and neurokinin B all significantly reduced the nerve-mediated contractile response in the presence of the endothelium at a concentration of 0.1 μM, with a rank order of potency substance P > neurokinin A > neurokinin B.
- 5 At a concentration of 0.1 μM, L-Pro also significantly reduced the nerve-mediated contractile response, unlike D-Pro and senktide.
- 6 It is concluded that the relaxation of the rabbit mesenteric artery produced by substance P is mediated by neurokinin-1 receptors (NK-1) located on the endothelium. Furthermore, of the analogues, L-Pro was particularly potent for these receptors.

Introduction

The tachykinins are a family of biologically active peptides that share the common C-terminal sequence Phe-X-Gly-Leu-Met-NH₂ (Erspamer, 1981). Substance P was initially identified in 1931 (von Euler & Gaddum, 1931) and fairly recently two additional mammalian tachykinins were isolated, these being neurokinin A and neurokinin B (Kangawa *et al.*, 1983; Kimura *et al.*, 1983; Nawa *et al.*, 1984). The discovery of neurokinin A and neurokinin B led to further studies aimed at identifying pharmacological preparations in which substance P was not necessarily the most potent tachykinin. For example, the rabbit pulmonary artery has been found to be particularly sensitive to neurokinin A (D'Orléans-Juste *et al.*, 1985) and the rat portal vein to neurokinin B (Mastrangelo *et al.*, 1986).

It has been proposed that the actions of the different tachykinins are mediated by multiple receptors which have been classified as neurokinin-1 (NK-1) (SP-P), NK-2 (SP-E) and NK-3 (SP-N) subtypes (Watson *et al.*, 1983; Laufer *et al.*, 1985; Lee *et al.*, 1986; Henry, 1987). These receptors have been

defined according to the rank orders of potencies of the various tachykinins. At the NK-1 receptor, substance P is the most potent tachykinin (for example, dog carotid artery: Regoli *et al.*, 1984; 1987) while at the NK-2 receptor, neurokinin A is more potent than substance P or neurokinin B (for example rabbit pulmonary artery: Regoli *et al.*, 1987) and at the NK-3 receptor, neurokinin B is the most potent (for example, rat portal vein: Regoli *et al.*, 1987).

In the rabbit mesenteric artery, substance P elicits vasodilatation which is dependent on the presence of an intact endothelium (Zawadzki *et al.*, 1981). In the present study, an attempt has been made to classify the subtype of receptor responsible for eliciting this endothelium-dependent vasodilatation by use of not only the naturally occurring tachykinins substance P, neurokinin A and neurokinin B, but also some recently developed tachykinin analogues that have been shown to exert a degree of selectivity for the receptor subtypes (Lee *et al.*, 1986; Wormser *et al.*, 1986; Fletcher *et al.*, 1987; Iversen *et al.*, 1987).

Until recently, there has been a lack of potent and specific antagonists available to characterize the

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tachykinin receptors, meaning that the only method available was to compare the rank order of potency of the tachykinins. However, preliminary work has shown that the recently developed tachykinin analogues used in this study display a specific pattern of agonist potencies characteristic to the tachykinin receptors: $[\text{Glp}^6, \text{L-Pro}^9]\text{SP}_{6-11}$ (L-Pro) shows a degree of selectivity for the NK-1 receptor on the smooth muscle of the guinea-pig ileum and guinea-pig urinary bladder (Iversen *et al.*, 1987; Lee *et al.*, 1986); $[\text{Glp}^6, \text{D-Pro}^9]\text{SP}_{6-11}$ (D-Pro) shows selectivity for the NK-2 receptor on the rat vas deferens and hamster urinary bladder (Iversen *et al.*, 1987; Lee *et al.*, 1986), while N-succinyl- $[\text{Asp}^6, \text{MePhe}^8]\text{SP}_{6-11}$ (senktide) has been found to show a degree of selectivity for the neuronal NK-3 receptor in the myenteric plexus of the guinea-pig ileum (Wormser *et al.*, 1986). It has been proposed that the comparison of the relative potencies of these agonists may be useful in characterizing the specific tachykinin receptors; thus, their relative specificities in the rabbit mesenteric artery were ascertained in this study.

Methods

Preparation of material

Adult male New Zealand white rabbits (2.5–3.4 kg) were killed by a blow to the back of the head and exsanguination. Sections of the mesenteric artery (first or second branch) were excised and cleared of surrounding fatty tissue under a dissecting microscope. Care was taken to avoid excessive trauma to the vessels so as to preserve the endothelium (Furchgott & Zawadzki, 1980). Sections of isolated artery were cut into rings, approximately 5 mm in length. Some of these rings deliberately had the endothelium removed by pulling a silk thread through the lumen of the vessels.

Each ring was mounted horizontally under isometric conditions in 20 ml organ baths by inserting two stainless steel wires through the lumen of the vessel, according to the method of Bevan & Osher (1972).

The tissues were bathed in Krebs solution of the following composition (mm): NaCl 133, KCl 4.7, NaH_2PO_4 1.35, NaHCO_3 16.3, MgSO_4 0.61, glucose 7.8, CaCl_2 2.52 (Bülbring, 1953). The Krebs solution was maintained at 37°C and bubbled with 95% O_2 and 5% CO_2 . Bovine serum albumin (0.005%) and bacitracin (30 mg l^{-1}) were added to the Krebs solution in order to prevent peptides adhering to the surfaces of the glassware and peptide degradation. Preparations were allowed to equilibrate for at least 1 h under a resting tension of approximately 0.75 g. Contractions of the circular smooth muscle were recorded by use of a Grass FT03C transducer and

displayed on a Grass ink-writing oscillograph (Model 79).

Pharmacology

A concentration-response curve was determined for noradrenaline (0.1–30 μM) on each segment in order to find the maximum contractile response. Relaxation to acetylcholine in segments in which tone was raised by noradrenaline (10 μM) confirmed whether the endothelium was present or absent (Furchgott & Zawadzki, 1980).

The vasodilator effect of the tachykinins: substance P (0.1 μM), neurokinin A (0.1 μM) and neurokinin B (0.1 μM) was tested in the presence and absence of the endothelium on preparations in which tone had been raised by noradrenaline (10 μM). Due to tachyphylaxis, the concentration of tachykinins could not be raised cumulatively, so separate doses were tested on segments. The tachykinins were washed out of the Krebs solution either once a maximum relaxant response had been reached or after the drug had been in contact with the vessel for approximately 2 min in the case of subthreshold doses. A non-cumulative dose-response curve could then be constructed.

In a second series of experiments, the specific agonists, L-Pro (0.1 μM), D-Pro (0.1 μM) and senktide (0.1 μM) were tested in a similar manner with the endothelium present and again a non-cumulative dose-response curve was constructed.

Nerve stimulation

The intramural nerves were electrically stimulated by two platinum wire electrodes placed parallel to and on either side of the vessel segment approximately 5 mm apart with a Grass S11 stimulator. Supramaximal voltage (60 V) and pulse duration (0.2 ms) were established and kept constant throughout the experiment. The vessel segments were stimulated by trains of pulses with a pulse interval of 32 ms for a period of 5 s which produced a measurable and reproducible response. With these parameters, the contractile response to nerve stimulation was completely blocked by tetrodotoxin (3 μM) and therefore appeared to be due to nerve stimulation and not to direct stimulation of the muscle. Stimulation was repeated every 30 s in order that the transient responses to the tachykinins and tachykinin analogues could be measured.

On each preparation, stimulations were repeated to ensure consistency of the response before a single concentration of substance P (0.1 μM), neurokinin A (0.1 μM) or neurokinin B (0.1 μM) was tested. Shortly after the maximum response had been attained (or approximately 2 min after addition of the drug to the Krebs solution in the case of subthreshold doses), the

Figure 1 Isolated transverse ring preparation of the rabbit mesenteric artery. (a, b) Non-cumulative concentration-response to substance P (SP) (0.01–100 nM) with tone raised and maintained by noradrenaline (NA) (10 μM), (a) in the presence of endothelium and (b) with endothelium removed. (c, d) Effect of SP on contractions produced by neurogenic transmural stimulation (0.2 ms; supramaximal voltage) for 5 s at 32 Hz in the presence (c) and absence (d) of endothelium.

Krebs solution was changed in order to minimize the tachyphylaxis of the tachykinins on the vessel. Similar experiments were carried out on sections of vessel with the endothelium absent.

The effect of the three tachykinin analogues on neurally-evoked contractions were then tested in a similar manner in a further series of experiments.

Drugs

Substance P, neurokinin A, neurokinin B, acetylcholine chloride and noradrenaline bitartrate were all obtained from Sigma Chemical Company. $[\text{Glp}^6, \text{L-Pro}^9]\text{SP}_{6-11}$, $[\text{Glp}^6, \text{D-Pro}^9]\text{SP}_{6-11}$ and $\text{N-succinyl-}[\text{Asp}^6, \text{MePhe}^8]\text{SP}_{6-11}$ were a gift from Merck, Sharp & Dohme. Neurokinin B was dissolved in dimethyl sulphoxide (DMSO). All the other

tachykinins and analogues were dissolved in 0.1% bovine serum albumin in distilled water. Noradrenaline was dissolved in 0.1 mM ascorbic acid. The noradrenaline was made up freshly each day. All tachykinins and analogues were stored at -20°C at a concentration of 0.1 mM in aliquots containing approximately 50 μl which were only defrosted once and used immediately.

Treatment of results and statistical methods

Relaxation to tachykinins and analogues were expressed as a percentage of the maximal relaxation to substance P in raised-tone preparations. Contractions to nerve stimulation were expressed as a percentage of the maximal contraction to noradrenaline in each preparation. In the figures, results are given

as a mean value calculated from a number of preparations denoted as *n* in the figure legends. Vertical bars indicate the standard error of the mean (s.e.mean). With the exception of substance P and L-Pro, the maximal relaxation to a particular tachykinin or analogue was not attained at the concentrations tested; thus, EC₅₀ values could not be calculated. Instead, drug potencies were expressed in terms of 40% of the maximal relaxation to substance P. Results from nerve stimulation experiments were analysed by Student's paired *t* test. A probability of *P* < 0.05 was considered significant.

Results

Rank order of potency of tachykinins and analogues in producing direct responses

Substance P (0.01–100 nM) produced concentration-dependent relaxations in isolated sections of rabbit mesenteric artery when applied in a non-cumulative manner to preparations with tone raised by 10 μ M noradrenaline (Figures 1a and 2a). These relaxations did not occur in the absence of the endothelium (Figure 1b). Presence of an intact endothelium was established by testing acetylcholine (0.1–300 μ M) on each preparation. A relaxant response of 80% or greater was taken as confirmation of the presence of the endothelium as shown by Furchgott & Zawadzki (1980). When the endothelium had deliberately been removed, the relaxant response to acetylcholine was also abolished.

Neurokinin A and neurokinin B (both 0.1–1000 nM) also produced concentration-dependent relaxations in the presence of the endothelium in raised-tone preparations. The overall rank order of potency was substance P > neurokinin A = neurokinin B (Figure 2a). As neither neurokinin A nor neurokinin B had reached maximal relaxation within the concentration range used (0.1–1000 nM), the EC₅₀ values could not be compared. Furthermore, as D-Pro and senktide did not cause relaxations equivalent to 50% of the maximal relaxation to substance P, EC₄₀ values were compared, the EC₄₀ value being the concentration of a particular tachykinin or analogue required to produce the equivalent of 40% of the maximal relaxation to substance P. The $-\log$ EC₄₀ for substance P (9.3 ± 0.13) was significantly different from the values calculated for neurokinin A (7.9 ± 0.09) and neurokinin B (7.7 ± 0.10). Substance P was therefore 20–40 times more potent than neurokinin A or neurokinin B, which were not significantly different from each other. Substance P, neurokinin A and neurokinin B did not produce a response at basal tone.

L-Pro, D-Pro and senktide also produced dose-

Figure 2 Isolated transverse ring preparation of the rabbit mesenteric artery with tone raised by noradrenaline (10 μ M). (a) Non-cumulative concentration-response curves to substance P (SP) (0.01–100 nM) (●) (*n* = 6), neurokinin A (0.1–1000 nM) (■) (*n* = 10) and neurokinin B (0.1–1000 nM) (▲) (*n* = 6). (b) Non-cumulative concentration-response curves to L-Pro (0.1–1000 nM) (●) (*n* = 10), D-Pro (1–1000 nM) (■) (*n* = 5) and senktide (1–1000 nM) (▲) (*n* = 6). In the presence of endothelium. Data points are means with s.e.mean shown by vertical bars.

dependent relaxations in preparations of the rabbit mesenteric artery which were dependent on the presence of the endothelium. Although neither D-Pro nor senktide reached maximal relaxation within the concentration range used (1–1000 nM), it was clear that L-Pro was more potent than either D-Pro or senktide, the rank order of potency thus being L-Pro > D-Pro = senktide (Figure 2b). Again the EC₄₀ values were calculated from the equivalent value found for substance P as neither D-Pro nor senktide reached maximal relaxant responses within the concentration-range used. The value calculated for $-\log$ EC₄₀ for L-Pro (7.9 ± 0.16) was significantly greater than that for D-Pro (6.3 ± 0.08) or senktide (6.2 ± 0.06). Thus L-Pro is in the order of 40–50 times more potent than D-Pro or senktide, which were not significantly different from each other.

Effects of tachykinins and analogues on neurogenic contractions of preparations

Substance P (0.1 μ M) caused a significant reduction in the contractile response to nerve stimulation in the presence of the endothelium (Figure 1c). There was no reduction in the contractile response to nerve stimulation in the absence of the endothelium (Figure 1d). Neurokinin A (0.1 μ M) and neurokinin B (0.1 μ M) also caused a significant reduction in the nerve-mediated contractile response in the presence of the endothelium (Figures 3a and 4a). The order of potency at the concentrations used was substance P > neurokinin A > neurokinin B (Figure 3a). As with substance P, neither neurokinin A nor neurokinin B produced a significant change in the neurally

Figure 3 Contractions of isolated transverse ring preparations of the rabbit mesenteric artery to 5 s periods of intramural nerve stimulation (0.2 ms, supramaximal voltage, 32 Hz) expressed as a percentage of the maximal contraction to noradrenaline. Contractions produced in the absence of any drug (control, open columns) are compared with those obtained in the presence of a tachykinin or analogue (hatched columns): 0.1 μ M substance P (SP) ($n = 8, 6$), neurokinin A (NKA) ($n = 6, 5$) or neurokinin B (NKB) ($n = 6, 5$) in the presence (a) and absence (b) of endothelium; and (c) in the presence of 0.1 μ M L-Pro ($n = 7$), D-Pro ($n = 5$) or senktide ($n = 5$) with endothelium present. Columns show means with s.e.mean shown by vertical bars. Significant differences (* $P < 0.05$) between control and experimental contractions were calculated by Student's paired t test.

induced contractile response when the endothelium was not present (Figure 3b).

At 0.1 μ M, L-Pro also caused a significant reduction in the contractile response to nerve stimulation, unlike D-Pro and senktide which had no effect (Figures 3c and 4b). In all three instances, the endothelium was present. In the absence of the endothelium, the inhibitory effect of L-Pro was not observed.

Figure 4 Contractions of isolated transverse ring preparations of the rabbit mesenteric artery to 5 s periods of intramural nerve stimulation (0.2 ms, supramaximal voltage, 32 Hz) expressed as a percentage change in contractile response between control and in the presence of (a) 0.1 μ M substance P (SP), neurokinin A (NKA) or neurokinin B (NKB) and (b) 0.1 μ M L-Pro, D-Pro or senktide. Endothelium present. Columns show means with s.e.mean indicated by vertical bars.

L-Pro was very selective when compared with D-Pro and senktide in inducing an inhibitory response in nerve-mediated contractions (Figure 4b). Although substance P was more potent than neurokinin A and neurokinin B, the contrast in potencies was not as marked as the contrast between the tachykinin analogues (Figure 4a).

Discussion

The present findings confirm that, in the rabbit mesenteric artery, substance P appears to be acting solely via a substance released from the endothelium, as both direct and nerve-mediated contractile responses were only affected in preparations with an intact endothelium. This is in keeping with previous findings in which it has been reported that substance P acts via the release of endothelium-derived relaxing factor (EDRF) (Zawadzki *et al.*, 1981). Furthermore, the fact that substance P inhibited both the contractile response induced by noradrenaline and nerve stimulation to the same extent, and that the nerve-mediated contractile response was unaffected in the absence of the endothelium, suggests that the effect may be purely due to a change in smooth muscle contractility. This supports previous findings which have shown that, in the rabbit pulmonary artery, cat cerebral artery and the canine blood-perfused gracilis muscle, substance P does not have a prejunctional effect (Endo *et al.*, 1977; Edvinsson & Skärby, 1984; Kahan *et al.*, 1985).

The tachykinin analogues were less potent than substance P, neurokinin A or neurokinin B. It is suggested that the reduction in potency is attributable

to the decrease in the length of the peptide chain from the N-terminal end. Indeed, it has been shown, in the dog carotid artery, that the NK-1 receptors are more sensitive to the tachykinins than to their fragments (Regoli *et al.*, 1987). However, L-Pro was very selective in inhibiting neurally mediated contractions when compared with the analogues, unlike substance P which was not as selective when compared with the other tachykinins.

Substance P was more potent than the other tachykinins in both the direct responses of raised-tone preparations and in reducing the nerve-induced contractile response, which suggests that the NK-1 receptor is responsible for mediating this action. Indeed, in the neurally mediated contractile responses, substance P was found to be more potent than neurokinin A, which was in turn more potent than neurokinin B. This order of potency is in keeping with that proposed for the NK-1 receptor (Regoli *et al.*, 1987). In raised-tone preparations, substance P was again the most potent tachykinin while neurokinin A and neurokinin B appeared to have similar potencies.

The tachykinin agonist L-Pro was found to be significantly more potent than either D-Pro or senktide both in raised-tone preparations and inhibiting the nerve-induced contractile response. This provides further evidence that in the mesenteric artery the tachykinin receptors are the NK-1 type, as in previous studies on established models such as the guinea-pig ileum and rat salivary gland, L-Pro has been shown to be selective for the NK-1 receptor (Iversen *et al.*, 1987). The inactivity of D-Pro and senktide also demonstrates the lack of NK-2 and NK-3 receptors as they are known to be selective for NK-2 and NK-3 receptors respectively (Piercey *et al.*, 1985; Wormser *et al.*, 1986).

One limitation of the present procedure for measuring the relative potencies of the tachykinins and analogues used was that the maximum effect could not be found in all cases as such a high concentration of peptide would be required. This was particularly true for the analogues D-Pro and senktide, which were extremely weak within the concentration-range used. In the absence of maximum effects to neurokinin A, neurokinin B, D-Pro and senktide, the pD_2 values could not be calculated from the incomplete concentration-response curves. Thus the method used to compare drug potencies had to be by comparison of the EC_{40} values, this being the concentration of drug required to produce the equivalent of 40% of the maximum relaxant response to substance P. Nevertheless, it can still be said that the comparison of the potencies of the tachykinins and the analogues clearly demonstrates that the analogues provide a far more selective response.

Table 1 Comparison of agonist potencies on raised-tone preparations of the rabbit isolated mesenteric artery

	$-\log EC_{40}$	Relative potencies
(a) Mammalian tachykinins		
Substance P	9.3 ± 0.13 (6)	1.0
Neurokinin A	7.9 ± 0.09 (10)*	0.04
Neurokinin B	7.7 ± 0.10 (6)*	0.025
(b) Tachykinin analogues		
L-Pro	7.9 ± 0.16 (10)	0.04
D-Pro	6.3 ± 0.08 (5)†	0.001
Senktide	6.2 ± 0.06 (6)†	0.001

All values are given as mean $\pm$ s.e.mean with number of observations (*n*) in parentheses. Note that as the pD_2 value was not attained in all cases, the results are expressed as $-\log$ (40% maximal relaxation to substance P) ($-\log EC_{40}$). Relative potency (substance P = 1) is defined as the ratio of EC_{40} of substance P to the EC_{40} of peptides tested. * Significant difference ($P < 0.05$) between substance P values and other tachykinin values, calculated by Student's unpaired *t* test. † Significant difference ($P < 0.05$) between L-Pro values and other tachykinin analogue values, calculated by Student's unpaired *t* test.

Thus it appears that, in the rabbit mesenteric artery, there are NK-1 receptors located on the endothelium. This is in keeping with previous work which has also shown that the endothelial tachykinin receptors are of the NK-1 type, for example, in the guinea-pig basilar artery (Edvinsson & Jansen, 1987), the dog carotid artery (Regoli *et al.*, 1984; 1987) and the pig coronary artery (Gulati *et al.*, 1987). This is in contrast to the tachykinin receptors located on the vascular smooth muscle which are either of the NK-2 type, for example, the rabbit pulmonary artery (Regoli *et al.*, 1987) or the NK-3 type, for example, the rat portal vein (Mastrangelo *et al.*, 1986). The proposition that substance P acts via the endothelium is supported by work carried out by Stephenson *et al.* (1986) who have shown by autoradiographic techniques in the dog carotid artery that there are indeed binding sites for substance P on the endothelium.

The present results thus lead to the proposal that, in the rabbit mesenteric artery, substance P acts via NK-1 receptors on the endothelium to cause vasodilatation.

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