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A comparison of the effects of three substance P antagonists on tachykinin-stimulated [^{3}H]-acetylcholine release in the guinea-pig ileum

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1 The potencies of three tachykinin antagonists [D-Pro^4,D-Trp^{7,9,10}]SP$_{(4-11)}$, [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP$_{(1-11)}$ and [D-Arg^1,D-Trp^{7,9},Leu^{11}]SP$_{(1-11)}$ (spantide) against eledoisin were examined in the guinea-pig ileum myenteric plexus, where a continuous superfusion system was employed to examine evoked release of [^{3}H]-acetylcholine ([^{3}H]-ACh]); effects on mechanical activity of the preparations were also measured.

2 Eledoisin was chosen as the standard tachykinin agonist since the rank order of potency observed in evoking release was eledoisin, kassinin, substance P, physalaemin; on this basis is may be presumed that an 'SP-E' type receptor was involved in the release process.

3 The two undecapeptide antagonists both significantly reduced the response to eledoisin (10 nM) as assessed by both [^{3}H]-ACh release and mechanical activity which under these conditions was largely dependent on ACh release, and the response levels could be restored by increasing the concentration of eledoisin to 100 nM. The pA_2 values for the two antagonists were estimated as 5.3 for [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP$_{(1-11)}$ and 5.2 for [D-Arg^1,D-Trp^{7,9},Leu^{11}]SP$_{(1-11)}$. [D-Pro^4,D-Trp^{7,9,10}]SP$_{(4-11)}$ was markedly less potent with a pA_2 value of <4.8.

4 All three antagonists possessed considerable inherent stimulatory activity as measured both by [^{3}H]-ACh release and mechanical activity, [D-Pro^4,D-Trp^{7,9,10}]SP$_{(4-11)}$ being the most active in this respect, a 10 μM concentration producing 50% of the response seen with 10 nM eledoisin.

5 These findings are discussed both in relation to tachykinin receptor classifications and limitations in the use of such antagonists in the study of the role of tachykinins in neurotransmission.

Introduction

Immunohistochemical studies have shown substance P-like immunoreactivity exists in axons of the guinea-pig ileum myenteric plexus (Nilsson *et al.*, 1975; Schultzberg *et al.*, 1980), and substance P-like activity has been shown to be released on electrical stimulation from intrinsic neurones (Franco *et al.*, 1979), by using specific desensitization to detect its actions. More recently, substance P release from the myenteric plexus of guinea-pig ileum longitudinal muscle preparations has been demonstrated by measuring the levels of substance P-like immunoreactivity produced on electrical stimulation (Holzer, 1983; 1984; Gintzler *et al.*, 1983).

It is generally believed that there are two components to the mechanical response of the guinea-pig ileum to substance P; the first of these is a rapid contraction resistant to both atropine and tetrodotoxin (TTX) (Rosell *et al.*, 1977), which fades within about 20 s to the second component, a maintained plateau response that can be markedly reduced by tetrodotoxin, tropicamide and physostigmine (Holzer & Lembeck, 1980). Recently, direct evidence for the dose-related, TTX-sensitive, Ca^{2+}-dependent release of acetylcholine from the myenteric plexus, by substance P and related tachykinins, has been presented (Yau & Youther, 1982; Fosbraey *et al.*, 1984).

The rank order of potency for substance P and some related tachykinins in releasing acetylcholine from the myenteric plexus in the guinea-pig ileum has been shown to differ from the potency order observed for the rapid, direct, atropine-insensitive, effect of these analogues in this tissue (Fosbraey *et al.*, 1984). These two potency orders appeared to follow those described

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by Lee *et al.* (1982) as characteristic of two substance P receptor subtypes designated 'SP-E' and 'SP-P' respectively. The receptor mediating acetylcholine release is denoted as 'SP-E'-like since eledoisin is considerably more potent than substance P or physalaemin in contrast to the direct action on smooth muscle where the tachykinins have similar potency.

The present work aims to study further the receptor mediating acetylcholine release by examining the effects of three substance P antagonists on the actions of eledoisin alone. The three analogues used were [D-Pro^4,D-Trp^{7,9,10}]SP$_{(4-11)}$ (Mizrahi *et al.*, 1982), [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP$_{(1-11)}$ (Rosell *et al.*, 1983a) and [D-Arg^1,D-Trp^{7,9},Leu^{11}]SP$_{(1-11)}$, which has been termed spantide (Rosell *et al.*, 1983b). We have extensively studied the first two (Bailey *et al.*, 1983; 1985) with respect to their activities against the tachykinins in a variety of smooth muscle preparations.

Methods

Male guinea-pigs weighing 300–500 g were stunned, bled at the neck and the ileum removed. Longitudinal muscle strips with myenteric plexus attached were prepared by a method modified from Paton & Zar (1968). The strips were set up to record isotonic tension developed and incubated by re-perfusion (6 ml min^{-1} with Krebs solution containing [^{3}H]-choline (1 μM; 15 ci mmol^{-1}, Amersham International plc) for 1 h as previously described (Fosbraey & Johnson, 1982). The preparations were then washed by overflow perfusion (2 ml min^{-1}) for a further hour in Krebs solution containing hemicholinium-3 (20 μM) and gelatin (1 g l^{-1}, to reduce peptide adsorption to glass) (Cleugh & Gaddum, 1963), the perfusate being discarded. From this time point ($t = 0$) the flow was reduced to 1 ml min^{-1} and the perfusate collected by means of a fraction collector. There were 5 × 2 min fractions of spontaneous release, 10 × 1 min collections, and 3 × 5 min fractions of spontaneous output. The preparation was exposed to tachykinins from $t = 10$ min to $t = 15$ min, eledoisin, kassinin, physalaemin, or substance P being used, and the antagonists, when present, were introduced at $t = 8$ min and perfused till $t = 15$ min.

Experiments were always performed in a set of four matched tissues exposed in parallel. In the antagonist studies the four treatments were eledoisin alone (10 nM), eledoisin (10 nM) plus antagonist (10 μM), eledoisin (100 nM) plus antagonist (10 μM), and antagonist alone (10 μM). Some experiments were also performed with the four tachykinin agonists alone, in parallel, at the same concentration (10 nM).

This matched dosing protocol allowed calculation of a dose-ratio (x) in that set for the concentration of antagonist used (A = 10 μM) assuming a parallel displacement of the tachykinin dose-response line. The apparent dissociation constant (K_D) calculated from the relation $A/K_D = (x - 1)$ was, for convenience, expressed in pA_2 units (since $pA_2 = -\log K_D$). Complications to this approach are discussed.

At the end of each set of experiments the tissues were dried, weighed and solubilized in Soluene-350 (Packard). Aliquots of perfusate fractions, and also solubilized tissue were assayed for radioactivity by liquid scintillation spectrometry. The results were calculated and plotted as the fractional release of [^{3}H]-acetylcholine ([^{3}H]-ACh) with the aid of a Hewlett Packard HP-85 computer. Increase in output of [^{3}H]-ACh (for determination as [^{3}H]-ACh see Szerb, 1976; Wikberg, 1977) evoked by peptides was calculated from the 'area' above spontaneous levels interpolated by regression analysis from the values before and after exposure to peptide (Fosbraey & Johnson, 1982; Fosbraey *et al.*, 1984). In this way the increase in output above spontaneous levels during the period $t = 10$ to 20 min was calculated.

Materials

[D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP$_{(1-11)}$ and [D-Pro^4,D-Trp^{7,9,10}]SP$_{(4-11)}$ were obtained from Bachem, Switzerland or Peninsula, Laboratories, California, via Merseyside Laboratories, Merseyside, UK. Eledoisin and kassinin were purchased from Peninsula Laboratories, California, physalaemin from Beckman, Geneva. Substance P was obtained from Beckman, Geneva, or Sigma, Poole, Dorset. All peptides were dissolved in 0.1% acetic acid and stored in aliquots sufficient for a single experiment at −20°C. A gift of spantide from Dr K. Folkers (Austin, Texas, U.S.A.) is gratefully acknowledged.

Results

Typical effects of the four tachykinin agonists at the same concentration (10 nM) in a set of experiments on [^{3}H]-ACh output are compared in Figure 1 where it may be seen that eledoisin and kassinin are markedly more active than substance P or physalaemin. We have previously made estimates of their relative potency with respect to [^{3}H]-ACh output (Fosbraey *et al.*, 1984) pointing out that the rank order of potency corresponds to the 'E'-type receptor of Lee *et al.* (1982). The concurrently observed increase in tension in such preparations could result from direct or indirect actions of the tachykinins, but under the conditions of the present experiments the prolonged indirect component is favoured (see Fosbraey *et al.*, 1984) and so may be used as an independent estimate of the extent of evoked ACh release.

Considering now the effects of the antagonists on

Table 1 Comparison of the three antagonists tested against eledoisin-stimulated [^{3}H] output and related tension development

Antagonist		*Absolute measure*				*Normalised measure*			
		E8	*E8 + A5*	*E7 + A5*	*A5*	*E8*	*E8 + A5*	*E7 + A5*	*A5*
SPA1		11.6	8.49	11.21	3.49	1.00	0.76*	1.07	0.29
	(8)	(1.56)	(1.29)	(1.86)	(1.16)		(0.04)	(0.20)	(0.10)
SPA2		11.41	9.05	14.07	2.39	1.00	0.78	1.27	0.23
	(3)	(1.79)	(1.75)	(5.50)	(0.69)		(0.04)	(0.43)	(0.09)
SPA3		7.70	5.53	10.93	2.72	1.00	0.88	2.34	0.49
	(8)	(2.36)	(1.09)	(1.22)	(0.90)		(0.12)	(0.39)	(0.17)
Tested against tension development									
SPA1		1.88	1.37	1.55	0.79	1.00	0.64*	1.00	0.31
	(8)	(0.39)	(0.56)	(0.18)	(0.49)		(0.15)	(0.19)	(0.11)
SPA2		2.01	0.76	1.44	0.93	1.00	0.38*	0.73	0.45
	(3)	(0.07)	(0.16)	(0.20)	(0.34)		(0.08)	(0.12)	(0.15)
SPA3		2.12	2.52	2.11	0.74	1.00	1.74	1.68	0.80
	(8)	(0.56)	(0.40)	(0.43)	(0.21)		(0.39)	(0.75)	(0.36)

Table 1 shows the results of concurrent testing of the ability of antagonists to inhibit eledoisin-stimulated (^{3}H]-ACh output and related tension development. The identity of the antagonists is denoted: [D-Arg^1, D-Pro^2, DTrp^{7,9}, Leu^{11}]SP$_{(1-11)}$, SPA1; [DArg^1, D-Trp^{7,9}, Leu^{11}]SP$_{(1-11)}$, SPA2 (spantide); [D-Pro^4-D-Typ^{7,9,10}]SP$_{(4-11)}$, SPA3. Exposure to eledoisin alone (10^{-8} M), 'E8'; eledoisin (10^{-8} M) + antagonist (10^{-5}M), 'E8A5'; eledoisin (10^{-7} M) + antagonist (10^{-5} M), 'E7A5'; and antagonist alone (10^{-5} M), 'A5', in horizontally matched quartets of experiments allowed conversion of 'absolute measures into 'normalized' with respect to the effect of E8 in that quartet. Figures are shown with (*n*) and (s.e.mean) in parentheses, and * denotes significantly different from unity ($P<0.05$).

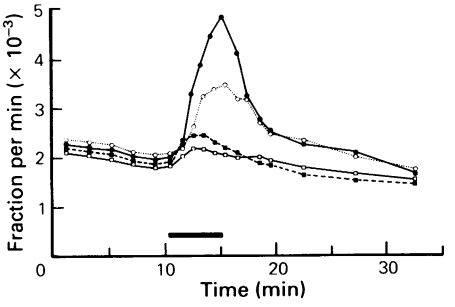

Figure 1 Release of [^{3}H]-acetylcholine ([^{3}H]-ACh) from four parallel preparations of longitudinal muscle exposed to one of the following tachykinins (10 nM) for 5 min (indicated by bar): eledoisin (●); kassinin (○); substance P (■) physalaemin (□).

this evoked ACh release using eledoisin as the standard, and most potent, agonist, it may be seen that they differed in their profiles of activity. [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP$_{(1-11)}$ reduced the [^{3}H]-ACh release evoked by 10 nM eledoisin and this was restored when the agonist concentration was increased tenfold. Though this is clear in absolute measures, comparison is made easier when the data are normalized in terms of the effects of standard eledoisin applications as described in the legend to Table 1. Further it is evident that [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP$_{(1-11)}$ alone evokes an increase in ^{3}H release of about 30% that of 10 nM eledoisin. This interpretation is fully confirmed by reference to the tension data which show a very similar trend overall.

Results from the experiments with the close structural analogue of [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP$_{(1-11)}$, [D-Arg^{1}D-Trp^{7,9},Leu^{11}]SP$_{(1-11)}$ (spantide), though fewer in number show a very similar profile in all respects.

However, the octapeptide [D-Pro^4,D-Trp^{7,9,10}]SP$_{(4-11)}$ failed to show a significant antagonism of either [^{3}H]-ACh output or the mechanical correlate. It may also be noted that the effects on ACh output of [D-Pro^4,D-Trp^{7,9,10}]SP$_{(4-11)}$ applied alone are more marked as estimated by either approach.

Discussion

The procedure adopted seems to give a reliable index of antagonist potency so long as care is taken to use a matched testing protocol. Though the prime index of ACh release is clearly obtained from [^{3}H]-ACh efflux the secondary measure, development of smooth muscle tone, does, under the special conditions of the experiments, lead to a very similar conclusion. It is important to note, however, that conventional short, rapid applications of tachykinins to this preparation would exert their actions via a quite different mechanism involving tachykinin receptors on the smooth muscle cells. It is of interest that these receptors have different properties from those resulting in ACh release and are, in fact, susceptible to blockade by the antagonists used here (Bailey *et al.*,1983; 1985) including [D-Pro^4,D-Trp^{7,9,10}]SP$_{(4-11)}$ which had little activity on the neuronal receptors.

Table 2 Estimates of pA_2 values for the substance P antagonists against eledoisin-evoked [^{3}H]-acetylcholine ([^{3}H]-ACh) release

Antagonist	pA_2	n
[D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP$_{(1-11)}$ (95% confidence limits)	5.34 (5.1–5.5)	8
[D-Arg^1,D-Trp^{7,9},Leu^{11}]SP$_{(1-11)}$ (95% confidence limits)	5.20 (4.9–5.5)	3
[D-Pro^4,D-Trp^{7,9,10}]SP$_{(4-11)}$	<4.8	8

Regarding the properties of the antagonists, there seems little doubt that [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]SP$_{(1-11)}$ and spantide have very similar potencies in antagonizing eledoisin both with pA_2 values of about 5.3 and 5.2 (Table 2). They seem similar also in respect of their ability to release ACh, with 10 μM antagonist having about 30% of the activity of 10 nM eledoisin in this respect.

[D-Pro^4,D-Trp^{7,9,10}]SP$_{(4-11)}$ had decidedly less potency as an antagonist of eledoisin on this system. A pA_2 value of <4.8 is shown in Table 2 since there were three out of eight experiments where no significant shift in the dose-response curve was seen with 10 μM antagonist and a notional value of 4.0 was assigned as the highest likely true value to allow some sort of comparison with the other antagonists. The reason for the low pA_2 values observed relative to a number of smooth muscle preparations we have investigated (Bailey *et al.*, 1983; 1985) seems unlikely to result from diffusional barriers in such a thin preparation with exposed myenteric plexus or from metabolic breakdown considering that a superfusion system was employed.

Again the antagonist showed a marked ability to release [^{3}H]-ACh, having at 10 μM about half the effect of 10 nM eledoisin, and estimated by the mechanical correlate even more activity. It will be evident from the observations made regarding the abilities of all the antagonists to release ACh in the concentration-range showing antagonism, that they appear to have partial agonist activities in this system. This activity was not so marked in studies we have made on various smooth muscle preparations (see Bailey *et al.*, 1986; Dodge & Morton, 1984), but in the present system prevents the

use of higher concentrations of the antagonists and further, may well bias estimates of pA_2 values. If similar partial agonist activity is found extensively in other peripheral and central nervous system preparations this property may limit the usefulness of these particular antagonists.

Turning to the receptor classification aspects, the most interesting finding is that the absolute and relative pA_2 values of the antagonists are rather unusual and correspond amongst the various smooth muscle preparations we have studied most closely to the guinea-pig bladder, the significance of this is being discussed more fully in Bailey *et al.* (1986).

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