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Pharmacological characterization of a substance P antagonist, [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-substance P

Stephen P. Watson

MRC Neurochemical Pharmacology Unit, Medical Research Council Centre, Medical School, Hills Road, Cambridge CB2 2QH

1 The substance P antagonist [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-substance P produced parallel log dose-response curve shifts to both substance P and eledoisin on five *in vitro* smooth muscle preparations.

2 The slope values of Arunlakshana-Schild plots were not significantly different from unity suggesting that it acts as a simple competitive antagonist on all five preparations with an association constant (K_a) in the range of $0.3-1.5 \times 10^6$ M^{-1}.

3 The K_a value of the antagonist was always slightly greater when tested against eledoisin than against substance P; however, this difference appears too small to suggest that these two agonists are acting on different receptor sub-types.

Introduction

Recently a number of substance P antagonists have been described (Rosell & Folkers, 1982). They are all structural analogues of substance P, and are characterized by the presence of a number of D-amino acid substitutions in their sequence. The most extensively characterized antagonists are [D-Pro^2, D-Phe^7, D-Trp^9]-substance P, [D-Pro^2, D-Trp^{7,9}]-substance P and [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-substance P, and these have all been reported to be competitive antagonists on the atropinized guinea-pig ileum with association constants (K_a) of 5×10^4 M^{-1} (Folkers, Horig, Rosell & Bjorkroth, 1981), 1.26×10^6 M^{-1} (Hakanson, Horig & Leander, 1982) and 2.04×10^6 M^{-1} (Rosell, Bjorkroth, Xu & Folkers, 1983) respectively. Earlier reports of agonistic actions of these compounds on the guinea-pig ileum have subsequently been shown to be due either to the release of histamine from mast cells (Hakanson *et al.*, 1982), or to the release of acetylcholine from neurones in the gut wall (Hawcock, Hayes & Tyers, 1982).

Previous work in this laboratory has demonstrated two distinct rank orders of potencies of substance P and its structural analogues in a variety of *in vitro* smooth muscle preparations, and this led to the suggestion of the existence of multiple receptors for substance P (Watson, Sandberg, Hanley & Iversen, 1983). On SP-P systems, typified by the guinea-pig ileum, guinea-pig bladder and guinea-pig vas deferens, substance P and eledoisin are approximately equipotent and act at nanomolar concentrations, while on SP-E systems, typified by the rat vas deferens and rat duo denum, substance P is approximately 2 orders of magnitude less potent than eledoisin and is active only at micromolar concentrations. The present study was, therefore, undertaken to characterize the most potent of the antagonists, [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-substance P, on SP-P and SP-E test preparations.

The results of previous workers indicated that some selectivity might be expected. Growcott & Tarpey (1983) observed that [D-Pro^2, D-Trp^{7,9}]-substance P was more potent against eledoisin than against substance P on the guinea-pig ileum, guinea-pig bladder and guinea-pig vas deferens and suggested that this antagonist might be selective to SP-E receptors. Similarly, Rosell *et al.* (1983) observed that [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-substance P exhibited selectivity between eledoisin and substance P on the guinea-pig ileum, although in contrast to the results of Growcott & Tarpey, the antagonist was found to be more potent against substance P than eledoisin. Furthermore, this antagonist was unable to block the action of substance P on the hamster urinary bladder, and had a significantly greater K_a value against substance P on the guinea-pig ileum than on the rat bladder; these results were interpreted as evidence for the presence of three distinct receptor sub-types (Rosell *et al.*, 1983).

Methods

In vitro *bioassays*

Male Sprague-Dawley rats (200–300 g), male Syrian hamsters (100–150 g) and male Hartley white guinea-pigs (250–400 g) were used for this study. The guinea-pig ileum longitudinal smooth muscle, guinea-pig vas deferens, guinea-pig bladder, rat duodenum, rat bladder and hamster bladder were mounted in 2 ml organ baths and used as described by Watson *et al.* (1982). Atropine (1×10^{-5} M) and mepyramine (1×10^{-5} M) were present throughout in order to block possible indirect effects of the antagonist (see Introduction).

An initial dose-response curve to substance P or eledoisin was determined using a 4 min time cycle. Subsequent dose-response curves were then determined in the presence of increasing concentrations of the antagonist [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Lei^{11}]-substance P, followed by a final recovery dose-response curve determined 1 h after the antagonist had been washed out. Each time a new concentration of antagonist was used, the tissue was exposed to this dose for a 10 min equilibration period and the dose-response curve was then determined using a 4 min cycle with the antagonist being given 3 min before the agonist. The concentrations of antagonist used were 1.66×10^{-6} M, 5.53×10^{-5} M and 1.66×10^{-5} M (on the guinea-pig ileum a further concentration of 5.53×10^{-7} M was used against eledoisin). All dose-response curves were evaluated using at least 6 doses of agonist.

Analysis of results

The initial and recovery log dose-response curves were averaged, and log dose-response curve shifts were estimated at the level of the EC_{50} value (i.e. the concentration of agonist required to produce 50% of the maximum response). Subsequently, data were pooled from individual experiments and analysed using an Arunlakshana-Schild plot (Arunlakshana & Schild, 1959); lines were fitted by linear regression.

Metabolism studies

The longitudunal muscle from the whole length of the guinea-pig ileum was cross-chopped at 350 μM on a MacIlwain tissue chopper and incubated at 37°C for 30 min in 5 ml Krebs solution; 3 washings, each with 3 × 5 ml Krebs, were performed during this period. The slices were then allowed to settle under gravity, and 25 μl portions were subsequently pipetted into 215 μl Krebs solution contained in small vials. Peptides were then added in 10 μl volumes and the tubes left at 37°C in a shaking water bath for 1 h. The tubes were subsequently transferred to a boiling water bath for 10 min and aliquots were then analysed on the guinea-pig ileum and compared to control samples. The concentration of peptide present in the incubation fluid could then be obtained from log dose-response curves for that peptide on the ileum. Experiments were performed in quadruplicate.

Materials

Substance P (molecular weight (mol. wt.) = 1348), eledoisin (mol. wt. = 1189) and [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-substance P (mol. wt. = 1498) were purchased from Peninsula Laboratories, California, and dissolved in distilled water and stored as aliquots at −20°C. The exact concentration of [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-substance P was determined by amino acid analysis and found to be 73% of that expected from its weight, and so all results have been corrected for this. Dilutions were made on the day of the experiment in Krebs solution containing 1% bovine serum albumin, and all solutions were kept on ice. Atropine sulphate and mepyramine maleate were obtained from Sigma, Poole. All other reagents were of analytical grade.

Results

Action of [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-substance P

[D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-substance P did not produce a contractile response on any of the five smooth muscle preparations at a concentration of 1.66×10^{-5} M. However, it did produce a reversible parallel shift in the log dose-response curves to both substance P and eledoisin on all five preparations, and these results are exemplified by its action on the guinea-pig ileum longitudinal smooth muscle as shown in Figure 1. The highest dose of antagonist used, 1.66×10^{-5} M, produced a 9–30 fold shift in the log dose-response curves to substance P or eledoisin.

The estimated K_a and slope values derived from the Arunlakshana-Schild plots are shown in Table 1. None of the slope values differ significantly from unity, indicating that [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-substance P is a simple, competitive antagonist against both substance P and eledoisin on all five preparations. The K_a values were in the range of $0.5–2.0 \times 10^6$ M^{-1}, but the value determined against eledoisin for each muscle was always slightly greater than the corresponding value determined against substance P (Figure 2; Table 1).

In contrast, [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-substance P at a concentration of 1.66×10^{-5} M did not antagonize the response to substance P or

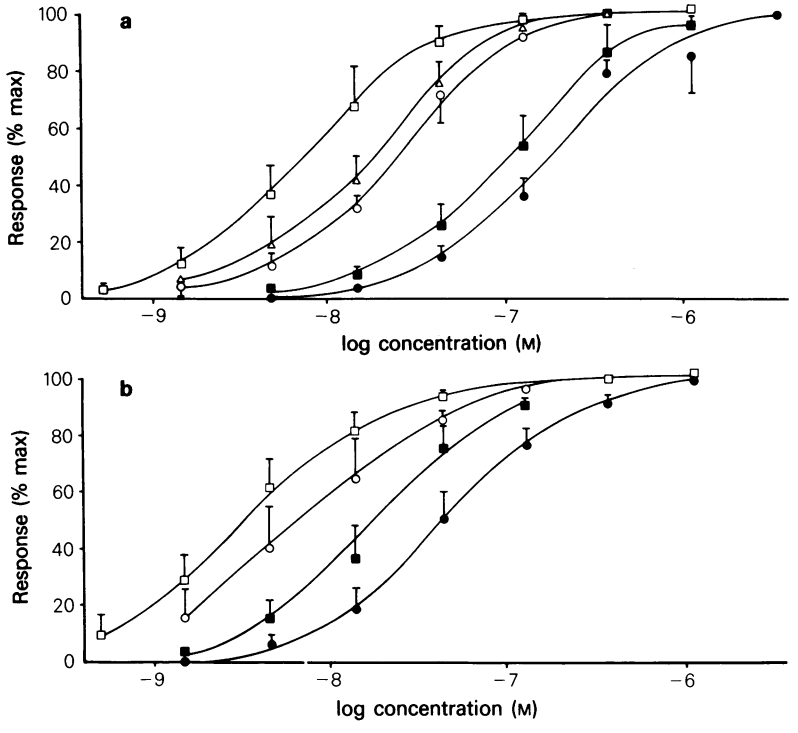

Figure 1 Antagonism of the contractile action of substance P and eledoisin on the guinea-pig ileum longitudinal muscle by [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-substance P: (a) eledoisin; (b) substance P. Each point represents the mean of 3 experiments; s.e.mean shown by vertical lines. Lines have been fitted by eye. (□) No antagonist; (△) 5.53×10^{-7} M antagonist; (○) 1.66×10^{-6} M antagonist; (■) 5.53×10^{-6} M antagonist; (●) 1.66×10^{-5} M antagonist.

Table 1 Antagonist properties of [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-substance P

Preparation	*Agonist*	*Log association constant* (95% confidence limits)	*Slope of Schild plot* ± s.e.mean
Guinea-pig ileum	Substance P	5.68 (5.55–5.92)	1.04 ± 0.14
	Eledoisin	6.11 (5.89–6.64)	1.04 ± 0.18
Guinea-pig bladder	Substance P	5.72 (5.42–6.81)	0.90 ± 0.25
	Eledoisin	5.89 (5.63–6.51)	1.21 ± 0.24
Guinea-pig vas deferens	Substance P	5.78 (5.61–6.05)	0.94 ± 0.12
	Eledoisin	5.96 (5.77–6.28)	1.04 ± 0.13
Rat duodenum	Substance P	5.71 (5.57–6.26)	0.93 ± 0.17
	Eledoisin	5.88 (5.71–6.16)	0.97 ± 0.12
Rat vas deferens	Substance P	5.56*	—
	Eledoisin	5.93 (5.79–6.13)	1.03 ± 0.09

Dose-response curve shifts were analysed at the EC_{50} level using at least 3 concentrations of antagonist per tissue. Data from three such experiments were pooled and analysed by the method of Arunlakshana & Schild (1959); lines were fitted by linear regression. *For the rat vas deferens with substance P as the agonist however, only one concentration of antagonist was used, 5.53×10^{-6} M, and the association constant (K_a) estimated from the equation

$$K_a = \frac{\text{dose ratio} - 1}{\text{concentration of antagonist}}$$

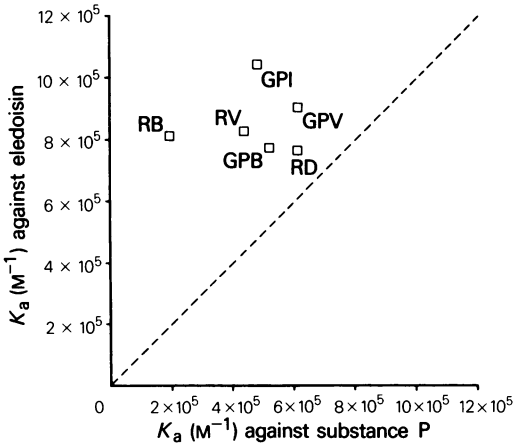

Figure 2 Correlation graph of the association constants (K_a) of [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-substance P against substance P and eledoisin on various smooth muscles. The dashed line represents $y = x$. Data from Table 1, except for the rat bladder for which the data were taken from Rosell *et al.* (1983). GPI = guinea-pig ileum longitudinal smooth muscle; GPB = guinea-pig bladder; GPV = guinea-pig vas deferens; RB = rat bladder; RV = rat vas deferens; RD = rat duodenum.

eledoisin on the hamster bladder, even when given 30 min before the agonist. Thus, this study has confirmed the observation previously made by Rosell *et al.* (1983).

Influence of [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-substance P on the metabolism of substance P and eledoisin by guinea-pig ileum longitudinal muscle slices

Aliquots of the fluid obtained after a 60 min incubation of substance P with 25 μl of 350 μM cross-chopped slices of guinea-pig ileum longitudinal smooth muscle had 4.4 ± 1% ($n = 3$) the potency of control samples as assayed on the guinea-pig ileum. Indeed, the metabolism of substance P in this time period may have been even greater as part of the activity observed on the ileum may be due to the presence of bioactive metabolites. This value was increased to 6.02 ± 1.1% ($n = 3$) when 6 μM of [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-substance P was present. In contrast, aliquots of the incubation fluid obtained from identical incubation tubes containing 2 μM eledoisin had 29.2 ± 3.5% the potency of controls. This value was increased to 38.5 ± 4.5% ($n = 3$) when 6 μM of [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-substance P was present.

Discussion

The present study has demonstrated that [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-substance P is a competitive antagonist to both substance P and eledoisin in five smooth muscle preparations. Further, this study has confirmed the observation originally made by Rosell *et al.* (1983) that this antagonist is not active against substance P or eledoisin on the hamster bladder, and the possibility therefore arises that this tissue contains a novel sub-type of substance P receptor.

The parallel shifts of the log dose-response curves to eledoisin and substance P produced by [D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11]]-substance P are in contrast to the results obtained by Rosell *et al.* (1983) who found that this antagonist produced non-parallel shifts to eledoisin on both the guinea-pig ileum and the rat urinary bladder and yielded slope values of Arunlakshana-Schild plots which were significantly different from 1.00. The explanation for these discrepancies may be related to the different experimental designs employed in the two studies. Thus, Rosell *et al.* (1983) allowed only a 2 min pre-incubation with the antagonist before the cumulative determination of the dose-response curve to the agonist. The cumulative approach for determining dose-response curves is open to criticism in that the response observed is influenced by the onset of desensitization which occurs after relatively short exposure (seconds) to substance P or eledoisin, and it has been reported that both eledoisin and substance P respond differently to desensitization on the guinea-pig ileum (Lee, Iversen, Hanley & Sandberg, 1982). Further, in the present study, it was often found that the pre-incubation of 2 min with the antagonist was insufficient to allow full onset of its action; generally, an incubation period of 4–10 min was necessary, depending on the muscle under study.

The K_a values for the antagonist against either substance P or eledoisin were similar on all five smooth muscle preparations demonstrating that the antagonist does not appear to differentiate between SP-P and SP-E systems. However, the K_a value determined against eledoisin on a particular muscle was always slightly larger than the corresponding value determined against substance P. This observation is very similar to that made by Growcott & Tarpey (1983) using the antagonist [D-Pro[2], D-Trp[7,9]]-substance P, and they interpreted their results as evidence that this antagonist was selective to the SP-E type of receptor. However, this does not appear to be the complete explanation for the present results, as different K_a estimates were obtained against substance P and eledoisin even on the rat vas deferens, which seems to contain only SP-E receptors (Watson *et al.*, 1983).

The rather small differences in the K_a estimates obtained against substance P and eledoisin appear to argue against the idea that the two agonists act on different receptors. Instead, alternative explanations for this slight difference should be considered, and this topic has been well reviewed by Kenakin (1982). For example, one possible explanation for such differences is that the antagonist selectively protects one of the agonists against degradation in the tissue biophase. This might have the effect that as the concentration of antagonist is increased, less metabolism of the agonist takes place and so the agonist is able to reach a higher concentration in the tissue biophase for a given bath concentration and so an artefactually smaller shift in the dose-response curve is observed. Conclusive evidence that such a hypothesis is the explanation for the present results is lacking, but it is interesting to note that rapid degradation of substance P in the biophases of the rat vas deferens and guinea-pig ileum longitudinal smooth muscle has been reported (Watson, 1983), and that the present study has shown that [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-substance P appears to decrease substance P metabolism. Further, although none of the Arunlakshana-Schild slopes for each tissue was significantly different from unity, there was a tendency for the value obtained against substance P, unlike that for eledoisin, to be less than 1.00.

Thus, in conclusion, it can be said that [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-substance P is a competitive antagonist that does not appear to select between SP-E and SP-P receptor sub-types. However, its inability to exhibit antagonism on the hamster bladder indicates that this tissue may contain a novel sub-type of substance P receptor.

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