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An examination of the pharmacology of two substance P antagonists and the evidence for tachykinin receptor subtypes

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1 The potencies of two tachykinin antagonists [D-Pro^4, D-Trp^{7,9,10}]-SP$_{(4-11)}$ and [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-SP$_{(1-11)}$ against four tachykinins were examined in a range of smooth muscle preparations, including guinea-pig ileum and bladder and rat colon muscularis mucosae and duodenum.

2 Parallel shifts in the log dose-response curves of all the tachykinins tested were observed in all tissues, except in the case of the guinea-pig bladder where [D-Pro^4, D-Trp^{7,9,10}]-SP$_{(4-11)}$ was without effect at concentrations up to 32 μM.

3 The slopes of the Schild plots for the two antagonists did not differ significantly from unity, with the exception of [D-Pro^4, D-Trp^{7,9,10}]-SP$_{(4-11)}$ in the rat duodenum, which may indicate a heterogeneous receptor population in this tissue. The antagonists displayed agonist selectivity in the case of the guinea-pig ileum where log dose-response curves to substance P and physalaemin were shifted less than those to eledoisin and kassinin.

4 Rank orders of potency for eledoisin, kassinin, physalaemin and substance P in the five preparations studied allowed classification of the tissues by the predominant receptor type according to the 'SP-P' and 'SP-E' scheme.

5 It is concluded that [D-Pro^4, D-Trp^{7,9,10}]-SP$_{(4-11)}$, in particular, displays tissue selectivity that may indicate different receptor populations, but classification of receptor and tissue types on this basis does not fully correspond with classifications based on agonist potencies. Such schemes should therefore be treated with caution at this stage.

Introduction

The modification of peptides by substitution of D-amino acids, or more bulky substituents, is an established method of achieving changes in biological activity. One of the earliest attempts to alter the activity of a tachykinin by this method was with a seven amino acid analogue of eledoisin (Schroder *et al.*, 1965), before the structure of substance P (SP) had been elucidated (Chang *et al.*, 1971). Recently a more concerted effort has been devoted to the synthesis of such analogues and their screening as potential tachykinin antagonists (Folkers *et al.*, 1981; Caranikas *et al.*, 1982) and findings with some of the earlier examples have been reviewed by Rosell & Folkers (1982). Many of these earlier analogues, however, still retained agonist activity in smooth muscle. In some cases this was attributable to histamine release (Fewtrell *et al.*, 1982; Håkanson *et al.*, 1982) though partial agonist activity at tachykinin receptors has been reported also (Hawcock *et al.*, 1982; Bailey & Jordan, 1984). The present work describes experiments with two more-recently reported tachykinin antagonists.

The first of these compounds, [D-Pro^4, D-Trp^{7,9,10}]-SP$_{(4-11)}$ described by Mizrahi *et al.* (1982) is based on the C-terminal octapeptide of substance P (SP) rather than the whole molecule, since it had been reported that this fragment is more potent than the parent molecule in several systems (Couture & Regoli, 1982). Thus, it was argued, an antagonist based on a

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fragment might show a correspondingly greater affinity for tachykinin receptors. Moreover, fragments which lack the N-terminal lysine and arginine residues of SP have little or no histamine-releasing activity (Fewtrell *et al.*, 1982). Furthermore [D-Pro^4, D-Trp^{7,9,10}]-SP$_{(4-11)}$ has been shown to antagonize the actions of SP and some of its fragments in a range of systems without exhibiting stimulant activity, (Regoli *et al.*, 1984a).

The second compound examined in the present study was [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-SP$_{(1-11)}$. It was originally described by Rosell *et al.* (1983b), and was reported as having antagonist activity against SP and other tachykinins in the rat urinary bladder and guinea-pig ileum whilst being devoid of agonist activity (Rosell *et al.*, 1983a). Recently, its antagonist effects in a number of other systems have been tested (Watson, 1983).

We have tested both of these compounds for their antagonistic effects against four tachykinins (SP, physalaemin (Phys), eledoisin (Ele) and kassinin (Kass) in several isolated smooth muscle systems in order to determine whether the relative affinities of antagonists could provide evidence to support or refute existing tachykinin receptor classifications.

Methods

Male Duncan Hartley guinea-pigs (150–450 g) and male Sprague-Dawley rats (150–250 g) were killed by stunning and exsanguination. Tissues were then prepared as follows.

Segments of guinea-pig whole ileum (GPI) or longitudinal muscle strip (GPILMS), sections of guinea-pig bladder detrusor muscle (GPB) prepared according to the method of Ambache & Zar (1970), rat duodenum (RDU) prepared by taking segments cut longitudinally from the gut wall, and rat colon muscularis mucosae (RCMM), prepared as described previously, (Bailey *et al.*, 1982), were set up in 1 ml silanised glass organ baths containing Krebs (GPILMS, GPB), Tyrode (GPI, RCMM) or de Jalon (RDU) solution. The bathing media were gassed with 95% O_2; 5% CO_2 and maintained at 32°C. Atropine (1 μM) was present throughout.

Contractions were recorded isometrically via Grass FTO3 transducers for GPILMS, GPB, RDU or isotonically for GPI and RCMM.

Having defined the full dose-response curve for the agonist under test, three-point dose-response curves were determined in triplicate. In most tissues a 10 min dosing cycle with a 30 s agonist contact time was employed. However, in the rat colon, agonist contact times of 1–2 min for SP, Phys and Ele and 5 min for Kass were necessary, and also, in the case of Kass, a 15 min dosing cycle. Three-point dose-response curves, encompassing the 50% response level, were then determined for the agonist in the presence of increasing concentrations of antagonist. Generally, antagonists were tested at 1, 3.2 or 10 μM and were added to the organ baths 7 min before the agonist dose. In the case of the guinea-pig ileum longitudinal muscle strip preparation, a shorter incubation time of 2 min was used for the octapeptide antagonist, since preliminary experiments had shown that dose-ratios measured at this time did not differ significantly from those measured after 7 min incubation.

The dose-ratios determined in the presence of each concentration of antagonist were used to produce Schild plots by means of an iterative, least squares, computer assisted method. Schild plots which had slopes not differing significantly from unity ($P < 0.05$) were replotted with slopes constrained to unity, according to the procedure described by Mackay (1978). From these plots, pA_2 values were estimated together with their 95% confidence limits.

In the case of the guinea-pig bladder, additional experiments were performed with [D-Pro^4, D-Trp^{7,9,10}]-SP$_{(4-11)}$ at 32 μM since it appeared relatively inactive in this preparation at lower concentrations. In addition, an estimate was made of its stability during contact with this tissue by incubating aliquots of the antagonist with the bladder preparation for 7 min, and then assaying the bathing medium for antagonist activity on the guinea-pig ileum longitudinal muscle strip.

Where estimates of relative activities of agonists were required, 3 + 3 bioassays were performed as previously described (Gater *et al.*, 1982) based on a randomized block design (Schild, 1942) with a minimum of three replicates for each dose. Relative activities and 95% confidence limits were established by analysis of variance as described by Colquhoun (1971).

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. Ele and Kass were purchased from Peninsula Laboratories, California, and physalaemin from Beckman, Geneva. Substance P was obtained from Beckman, Geneva, or Sigma, Poole, Dorset, UK. All peptides were dissolved in 0.1% acetic acid and stored in aliquots sufficient for a single experiment at −20°C. Additional gifts of eledoisin and physalaemin from Dr R. de Castiglione (Farmitalia, Milan) are gratefully acknowledged.

Results

Both antagonists produced parallel shifts in the log dose-response curves for all agonists in each of the five

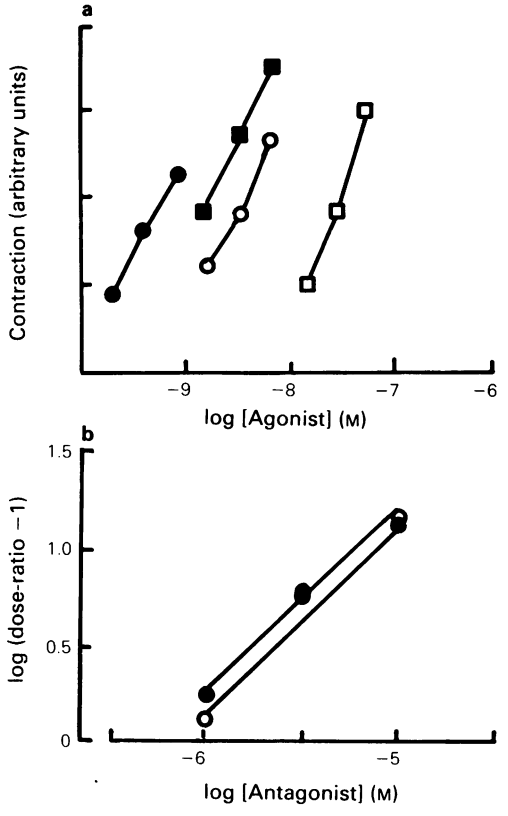

Figure 1 (a) Dose-response curves for substance P (SP, ●) and eledoisin (Ele, ■) from typical experiments in the guinea-pig ileum in the absence (solid symbols) and presence (open symbols) of [D-Pro^4, D-Trp^{7,9,10}]-SP$_{(4-11)}$, 10 μM. (b) Schild plots for SP (●) and Ele (○) from experiments with [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-SP$_{(1-11)}$ in the rat duodenum.

preparations, except in the case of the octapeptide antagonist on the guinea-pig bladder where no significant antagonism was observed even at a concentration of 32 μM.

Examples of log dose-response curves for SP and Ele in the guinea-pig ileum, determined in the absence and presence of the octapeptide antagonist, are shown in Figure 1(a), from which it can be seen that this compound exhibits agonist selectivity in that the lateral shift is greater for Ele than for SP. From the results of experiments of this type and using several concentrations of antagonist, Schild plots were constructed to allow estimation of pA_2 values and their confidence limits (Table 1). The slopes of the Schild plots did not differ significantly from unity except in the case of the octapeptide antagonist in the rat duodenum, for which the observed slopes are listed. In all other cases, the pA_2 estimates were derived from plots with a constrained slope of unity (see legend).

Figure 1(b) shows such Schild plots for the undecapeptide antagonist in the rat duodenum, where no marked agonist selectivity was observed.

Antagonist selectivity was most marked in the guinea-pig bladder preparation where, in contrast to the undecapeptide antagonist, the octapeptide antagonist lacked any activity in the concentration range tested. Biodegradation of the peptide did not seem to be responsible since, following incubation of a 10 μM solution with segments of bladder for 7 min, the remaining bath concentration was approximately 4 μM (as assayed on a guinea-pig ileum strip preparation), or equivalent to 19 μM after incubation of the highest concentration tested, (32 μM).

In view of the actions of earlier antagonists, it is worth noting that the only instance of apparent agonist activity of the antagonist compounds in this study was seen with [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-SP$_{(1-11)}$, which produced occasional stimulant activity in the guinea-pig bladder at a concentration of 10 μM.

Discussion

Initial proposals of multiple tachykinin receptor subtypes based on relative potencies of agonists (Teichberg *et al.*, 1981; Lee *et al.*, 1982), have been followed by studies employing a range of putative tachykinin antagonists which have yielded some surprising results. For example, [D-Pro^2, D-Phe^7, D-Trp^9]-SP$_{(1-11)}$ (Hawcock *et al.*, 1982), was suggested as being a selective agonist at a subclass of tachykinin receptors in the guinea-pig ileum. A closely related analogue, [D-Pro^2, D-Trp^{7,9}]-SP$_{(1-11)}$, (Growcott & Tarpey, 1983), was reported to be more potent on the 'SP-E' as opposed to the 'SP-P', receptor in the classification proposed by Lee *et al.*, (1982) since, on some tissues, it was more active against eledoisin than SP.

Our preliminary studies with [D-Pro^4, D-Trp^{7,9,10}]-SP$_{(4-11)}$ have demonstrated that it had apparent tissue- and agonist selectivity which did not seem to correspond directly with the 'SP-P' and 'SP-E' receptor classification (Bailey *et al.*, 1983). [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-SP$_{(1-11)}$ has been the subject of conflicting reports. Rosell *et al.*, (1983b) claimed that it distinguished between SP and eledoisin in the guinea-pig ileum, whereas Watson (1983) found no significant agonist selectivity in guinea-pig ileum longitudinal muscle strips. However, both agreed that this analogue lacked activity in the hamster bladder, which was therefore suggested to contain a novel tachykinin receptor sub-type.

In the light of the reports of Watson (1983) and Rosell *et al.*, (1983b) concerning the hamster bladder,

Table 1 pA_2 values and 95% confidence limits obtained with two agonists in various smooth muscle preparations

Preparation	*Agonist*	*[D-Pro⁴, D-Trp⁷,⁹,¹⁰]-SP(4-11)* pA_2 *(95% confidence limits: n)*	*[D]-Arg¹, D-Pro², D-Trp⁷,⁹, Leu¹¹] SP(1-11)* pA_2 *(95% confidence limits: n)*
GPI	SP	5.80 (5.71, 5.88: 5)	5.67 (5.48, 5.87: 4)
	Phys	6.12 (6.04, 6.19: 5)	6.11 (5.90, 6.32: 4)
	Ele	6.30 (6.20, 6.39: 5)	6.50 (6.36, 6.64: 4)
	Kass	6.38 (6.29, 6.48: 5)	6.43 (6.35, 6.50: 4)
GPILMS	SP	5.95 (5.84, 6.05: 5)	5.70 (5.45, 5.95: 5)
	Phys	5.89 (5.75, 6.04: 5)	5.90 (5.64, 6.16: 5)
	Ele	6.54 (6.50, 6.77: 5)	6.01 (5.81, 6.20: 6)
	Kass	6.35 (6.06, 6.64: 5)	6.11 (5.92, 6.31: 4)
RCMM	SP	5.58 (5.49, 5.66: 4)	5.66 (5.58, 5.74: 4)
	Phys	5.57 (5.51, 5.63: 4)	5.64 (5.48, 5.81: 4)
	Ele	5.46 (5.36, 5.55: 4)	5.79 (5.74, 5.84: 4)
	Kass	5.53 (5.42, 5.63: 4)	5.65 (5.56, 5.73: 4)
RDU	SP	5.73 (4.95, 6.50: 5) b = 0.517	6.21 (5.95, 6.47: 4)
	Phys	5.96 (4.61, 6.52: 5) b = 0.231	6.34 (6.09, 6.59: 5)
	Ele	6.00 (5.13, 6.87: 5) b = 0.426	6.26 (5.90, 6.62: 4)
	Kass	5.38 (4.78, 6.00: 5) b = 0.485	5.96 (5.59, 6.32: 4)
GPB	SP	<4.5 ($n = 6$)	6.12 (5.96, 6.28: 5)
	Phys	<4.5 ($n = 6$)	5.89 (5.72, 6.06: 5)
	Ele	<4.5 ($n = 6$)	5.90 (5.67, 6.13: 5)
	Kass	<4.5 ($n = 6$)	6.06 (5.88, 6.24: 5)

SP = substance P, Phys = physalaemin, Ele = eledoisin, Kass = kassinin. The slopes of the Schild plots for the octapeptide antagonist in the rat duodenum are included since they differed significantly from unity ($P < 0.05$).
GPI = guinea-pig ileum; GPILMS = guinea-pig ileum longitudinal muscle strip; RCMM = rat colon muscularis mucosae; RD = rat duodenum; GPB = guinea-pig bladder.
b = the estimated regression coefficient of the Schild plot where this was found to differ significantly from unity.

it may be significant that, in the present comparative study, the guinea-pig bladder is the only smooth muscle tissue in which the two antagonists show markedly different activities. This suggests that the tachykinin receptor population in this preparation may differ from those in the other tissues studied. Despite the substantial loss of activity (approximately 60%) of the octapeptide antagonist following incubation for 7 min with the bladder preparation, this cannot explain its lack of effect since a sufficiently high concentration remained to inhibit the effects of tachykinins in the guinea-pig ileum longitudinal muscle strip. Furthermore, the octapeptide and undecapeptide antagonists possess similar paradoxical relative activities in a continuous superfusion system, namely tachykinin-stimulated release of [^{3}H]-acetylcholine from the myenteric plexus of the guinea-pig ileum, in which limiting factors such as diffusion and metabolism should be minimized (Featherstone *et al.*, 1986).

Some previous reports have taken non-unity slopes of Schild plots or agonist selectivity of tachykinin antagonists as evidence of multiple receptor subtypes within a given preparation (Briggs *et al.*, 1983; Growcott & Tarpey, 1983; Rosell *et al.*, 1983a). This situation arises in the present study and it is worth considering whether there may be alternative explanations for such observations.

The shallow slopes of Schild plots for the octapeptide antagonist in the rat duodenum could be interpreted as evidence for a heterogeneous receptor population (see, Furchgott, 1972; 1976). However, an alternative possibility is that the rate of inactivation of agonists may be affected by the antagonist, or that the high concentrations of agonist used in the presence of the antagonist in itself causes this effect, (for discussion, see Blinks, 1967; Furchgott, 1967; Kenakin, 1980). Until these alternatives can be ruled out, it may be unwise to conclude from the indicators described above that a given tissue contains more than one tachykinin receptor subtype, or to use the pA_2 intercept as an accurate estimate of the affinity constant.

In guinea-pig ileum segments and longitudinal muscle strip, both antagonists exhibited agonist selectivity without any clear deviation from unity slope in the respective Schild plots. However it has been

Table 2 Comparison of potencies of antagonists in tissues classified on the basis of relative activities of agonists

Preparation	*Relative activities of agonists* (SP = 1)				*Receptor group*	*Antagonist pA_2 values*				*Tissue group*
						Octapeptide		Undecapeptide		
	SP	*Phys*	*Ele*	*Kass*						
GPI[1]	1.00	2.28	0.64	0.44	P	6.30	6.90[5]	6.50		TK1
GPILMS[1]	1.00	2.07	0.89	0.97	P	6.54		6.01	6.11[6]	TK1
DCA[2]	1.00	1.00	0.33	0.15	P	6.30[5]				TK1
GPVD[3]	1.00	2.43	0.90	0.20	P			5.96[6]		TK1
RCMM[1]	1.00	0.26	8.40	59.00	E	5.46		5.79		TK2
RMV[2]	1.00	1.10	2.10	3.21	E	5.42[5]				TK2
GPT[2]	1.00	1.30	4.20	13.50	E	5.70[5]				TK2
RVD[3]	1.00	0.60	80.00	160.00	E			5.93		TK2
RDU[1]	1.00	4.60	5.75	16.61	E	6.00		6.26	5.88[6]	TK2
GPB[1]	1.00	5.49	4.64	1.79	P/E	<4.5		5.90	5.89[6]	TK3
GPIMP[4]	1.00	0.40	26.55	2.65	E	<4.8		5.34		TK3

Tissues are classified according to the proposals of Lee *et al.*, (1982) on the basis of relative activities of agonists (Receptor group). The tissues have been further sub-divided in terms of pA_2 values obtained for [D-Pro[4], D-Trp[7,9,10]]-$SP_{(4-11)}$. Results from the present study and from published reports in the literature are included.
GPI = guinea-pig ileum; GPILMS = guinea-pig ileum longitudinal muscle strip; DCA = dog carotid artery; GPVD = guinea-pig vas deferens; RCMM = rat colon muscularis mucosae; RMV = rabbit mesenteric vein; GPT = guinea-pig trachea; RVD = rat vas deferens; RDU = rat duodenum; GPB = guinea-pig bladder; GPIMP = guinea-pig ileum myenteric plexus.
Literature references: (1) Present authors; (2) Couture & Regoli (1982); (3) Iversen *et al.* (1981); (4) Featherstone *et al.* (1986); (5) Regoli *et al.* (1984a); (6) Watson (1983).

demonstrated that Schild plots with apparent slopes of unity can be obtained in the case where both the agonist and antagonist interact with two receptor types (Lemoine & Kaumann, 1983). Such plots consist of two lines with unity slope joined by a shallower inflection. Here again, it is possible to generate similar plots assuming one receptor site and a saturable inactivation process for the agonist (Furchgott, 1972).

Yet another explanation of agonist selectivity as displayed in the guinea-pig ileum preparations is discussed by Bailey (1985) on the basis of receptor-limited diffusion of agonist molecules in the tissue.

It is, of course, important that pA_2 estimates are independent of the exact experimental conditions used if they are to be taken as unique parameters of any given receptor: antagonist interaction, and, in this respect, it is encouraging that pA_2 estimates in the two guinea-pig ileal preparations agreed so well despite the differences in experimental protocols (see Methods).

Although it was not the primary purpose of this study to use rank orders of potencies of agonists as a means of classifying receptor subtypes, it was of interest to compare this approach, using the scheme devised by Lee *et al.* (1982), with a classification based on antagonist pA_2 values. To this end Table 2 lists estimates of relative potencies we and others have made (see legend) and, on this basis, gives a predominant receptor type of 'P' or 'E' using the criteria of Lee *et al.* (1982), for all preparations in the present study plus certain others of interest.

This classification is compared with antagonist pA_2 values in the tissues assigned to three groups TK1, TK2 and TK3 in descending pA_2 order for the octapeptide antagonist.

It is apparent from Table 2 that the pA_2 bands for the groups TK1 and TK2 correspond reasonably closely with the agonist 'P'- and 'E'-type classification. The two tissues in the TK3 group present more problems. They share the characteristic that the octapeptide is of markedly low activity, though the undecapeptide is as active as on some tissues in the TK2 group. With respect to agonist potency, the guinea-pig myenteric plexus preparation has an 'E'-type activity profile, but the guinea-pig bladder could be regarded as 'E'-type or 'P'-type.

It would be useful in the comparison of these two classification schemes if other approaches to identification of receptors were readily available. The effects of treatment with phenoxybenzamine might provide a useful test since responses to tachykinins in a number of systems are antagonized by this compound. Thus, phenoxybenzamine affects responses to a number of tachykinins on the guinea-pig ileum (Lin & Musacchio, 1983) (TK1 group) and to eledoisin on the guinea-pig bladder (Growcott *et al.*, 1983) (TK3 group), but not any of the standard tachykinin

agonists on the rat colon muscularis mucosae (Bailey, 1985) (TK2 group).

Similarly, if the tendency of tissues to show desensitization to the tachykinins is regarded as a receptor-, rather than mechanism-related characteristic, then it might also be used for the purposes of classification. Desensitization is marked in the guinea-pig ileum preparation (Jordan, 1980) and dog carotid artery (Couture & Regoli, 1982) (TK1 group), but it is also seen in the rabbit mesenteric vein (Couture & Regoli, 1982) (TK2 group) and the guinea-pig bladder (Sjorgen *et al.*, 1982) (TK3 group). However, the rat colon muscularis mucosae (Bailey *et al.*, 1982) (TK2 group), does not desensitize very readily.

It is clear that there are discrepancies in each of these classification schemes. Taken overall, the agreement between the two classical approaches of agonist and antagonist potency is quite good, and it would be premature to advance a three subtype scheme based on the antagonist studies. Perhaps the TK3 tissues would be better regarded as extra examples of 'E'-type receptors, especially since the other classification approaches discussed yield equivocal results.

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