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Conformationally Constrained Tachykinin Analogs Which Are Selective Ligands for the Eledoisin Binding Site

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SUMMARY

We have prepared a series of conformationally constrained hexapeptide analogs of substance P which are 500–1500-fold more potent as inhibitors of ^{125}I-labeled Bolton Hunter-conjugated eledoisin binding to rat brain cortex membranes than as inhibitors of ^{125}I-labeled Bolton Hunter-conjugated substance P binding. These analogs stimulate guinea pig ileum contraction (ED_{50} 1–16 nM) and stimulate rat vas deferens contraction (ED_{50} 2–4 μM). However, these peptides are poor stimulators of rat salivation (>40 nmol/100 g body weight). Thus, based on both their receptor potency and pharmacological potency, these peptides are potent and selective tachykinin analogs. These data indicate that a specific carboxyl-terminal conformation is recognized by the ^{125}I-labeled Bolton Hunter-conjugated eledoisin binding site and that this conformation is different from the conformation recognized by the ^{125}I-labeled Bolton Hunter-conjugated substance P binding site. Hexapeptides containing phenylalanine, isoleucine, and valine identical with the carboxyl-terminal sequences of substance P, eledoisin, and neurokinin B, respectively, were nearly equipotent as inhibitors of ^{125}I-labeled Bolton Hunter-conjugated eledoisin binding. The valine analog was only ~5-fold less potent than the isoleucine and phenylalanine analogs as an inhibitor of ^{125}I-labeled Bolton Hunter-conjugated substance P binding. Thus, unknown determinants in the amino-terminal sequences of substance P must strongly contribute to the carboxyl-terminal peptide selectivity and conformation. The contraction of guinea pig ileum induced by one of the conformationally constrained analogs is attenuated by pretreatment of the tissue with atropine (2 μM), while that induced by substance P methyl ester, a selective inhibitor of ^{125}I-labeled Bolton Hunter-conjugated substance P binding, is not. Thus, the constrained analog has a higher affinity for the tachykinin receptors in the guinea pig myenteric plexus which are responsible for acetylcholine release than for the tachykinin receptors present on the smooth muscle cells.

The mammalian neuropeptide substance P[1] and the amphibian peptide eledoisin are members of a family of peptides termed tachykinins which share the carboxyl-terminal sequence -Phe-X-Gly-Leu-Met-NH_2 and which cause contractions of various smooth muscle preparations. Since the rank order of potency of the tachykinins in various assay systems is different, Lee *et al.* (1) have proposed that there may be two subtypes of tachykinin receptors. Thus, in "P" type tissues, substance P and eledoisin are nearly equipotent, while in "E" type tissues, eledoisin is 10–100 times more potent than substance P. We have shown that ^{125}I-labeled Bolton Hunter-conjugated substance P and ^{125}I-labeled Bolton Hunter-conjugated eledoisin bind to pharmacologically distinct sites in rat brain cortex membranes (2). Autoradiography of rat brain slices incubated with these two ligands *in vitro* indicates that the two binding sites are distributed differently (3, 4). Thus, there are at least two distinct tachykinin receptors in the mammalian central nervous system.

Eledoisin is only 3% as potent as substance P as an inhibitor of ^{125}I-BH-SP binding to rat brain cortex membranes (5) and rat parotid cells (6). In contrast, eledoisin is 6.8 times more potent than substance P as an inhibitor of ^{125}I-BH-eledoisin binding to rat brain cortex membranes (2, 7). Neurokinin B (8, 9), a tachykinin recently isolated from porcine spinal cord, is at least 60-fold more potent as an inhibitor of ^{125}I-BH-eledoisin binding than as an inhibitor of ^{125}I-BH-SP binding (7). Therefore, the ^{125}I-BH-eledoisin binding site may be the receptor for the endogenous mammalian peptide, neurokinin B.

We previously showed that a substance P analog in which the carboxyl-terminal amide is replaced by a methyl ester is 30% as potent as substance P in stimulating rat salivation and

[1] Sequences of peptides for: substance P, Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2; eledoisin, pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2; neurokinin B, (also neuromedin K or neurokinin β), Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2.

ABBREVIATIONS: ^{125}I-BH-SP, ^{125}I-labeled Bolton Hunter-conjugated substance P; ^{125}I-BH-eledoisin, ^{125}I-labeled Bolton Hunter-conjugated eledoisin; <Glu, pyroglutamyl.

is equipotent to substance P in the guinea pig ileum assay (10). While substance P methyl ester is potent as a substance P-P agonist, it is only weakly active in E type assays (11). In addition, substance P methyl ester is 1200 times more potent as an inhibitor of ^{125}I-BH-SP binding than as inhibitor of ^{125}I-BH-eledoisin binding (7). Thus, substance P methyl ester is a selective agonist for receptors which bind ^{125}I-BH-subtance P.

The relative potencies of peptides to inhibit the binding of ^{125}I-BH-SP and ^{125}I-BH-eledoisin do not exactly correlate with the potencies of the peptides in either P or E type smooth muscle tissue preparations (7). Thus, while the concept of P and E tissue subtypes proposed by Lee *et al.* (1) has proven useful, it is likely that these tissues contain mixtures of the ^{125}I-BH-SP and ^{125}I-BH-eledoisin binding sites or other types of tachykinin receptors. Identification of more selective agonists and antagonists should facilitate the determination of the physiological roles of these receptor subtypes and their tissue distribution. We now describe conformationally restricted hexapeptide analogs of substance P which are selective agonists for the ^{125}I-BH-eledoisin binding site. The selectivity of these analogs demonstrates that the structural and conformational requirements for binding to the ^{125}I-BH-SP and ^{125}I-BH-eledoisin receptors are different.

Materials and Methods

Substance P, pyroglutamyl (6–11) substance P hexapeptide, and eledoisin were purchased from Peninsula Laboratories. Substance P methyl ester was purchased from Cambridge Research Biochemicals. Lactam-containing peptides were synthesized by methods described previously (12). All other peptides were synthesized by standard procedures.

^{125}I-BH-SP and ^{125}I-BH-eledoisin binding assays. ^{125}I-BH-SP and ^{125}I-BH-eledoisin were supplied by New England Nuclear and were prepared as described previously (3, 5). ^{125}I-BH-SP and ^{125}I-BH-eledoisin binding to rat parotid cells (6) and rat brain cortex membranes (5, 7) were measured as described previously. Briefly, ligands (0.5 nM, 600–1300 Ci/mmol) were incubated at 20° with cells (0.25 mg) or membranes (0.1 mg) in 0.15 ml 0.05 M Tris, pH 7.5, containing bovine serum albumin (200 μg/ml), chymostatin (50 μg/ml), and $MnCl_2$ (1 mM). The assay was terminated by filtration on Whatman GF/F filters which had been soaked overnight in 0.1% polyethyleneimine.

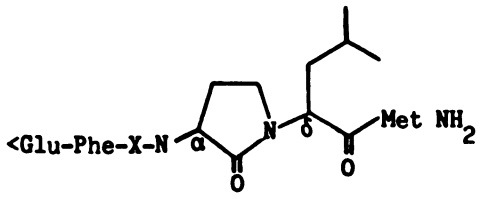
Biological assays. Salivation assays were performed using Charles River Sprague-Dawley CD rats (200–300 g) as described previously (6). Briefly, compounds were injected into the tail vein of ether-anesthetized animals and saliva was collected for 2 min from the buccal cavity. Each point in a titration curve was done in triplicate.

The guinea pig ileum contraction assay was performed as described previously (10). Briefly, guinea pig ileum strips (~2 cm) were incubated under 40 mm Hg resting tension in 5 ml of Krebs-Ringer bicarbonate, pH 7.4, at 37°. Peptides were diluted into buffer containing bovine serum albumin (0.2 mg/ml) and were added in 30-μl aliquots.

The rat vas deferens assay was performed using tissue obtained from Sprague-Dawley rats (200–350 g), mounted in 3-ml siliconized glass organ baths containing Krebs-Henseleit solution at 37°. Electrical field stimulation was with single, biphasic pulses (Grass S88) delivered at 0.15 Hz between platinum electrodes at the top and bottom of the bath (voltage drop, 11.5 v/cm) using a supramaximal voltage (bath current, 70–80 mamp). The values for ED_{50} were obtained using the ALLFIT four-parameter logistic model and are geometric means of three to four observations except for eledoisin, where $n = 20$.

Results

The glycine residue at position 9 of substance P is an important determinant of tachykinin activity. Since glycine has no side chain, its role is probably conformational. Glycine is often found in turn conformations, and incorporation of a lactam ring of appropriate size bridging between Gly[9] and Leu[10] (12, 13) is one way to test for turns in the receptor-bound conformation. Based on these concepts, hexapeptide analogs containing α-N'-cyclo[2]dipeptide units (ANC-2)[2] having either the *R*- or *S*-configuration (Fig. 1) at the lactam α-carbon were prepared. A hexapeptide sequence was chosen as the basis of analog design because of the known high potency of this fragment as a substance P agonist in many smooth muscle assays (14).



IA) X = Phe (configuration δ = S, α = R)

B) X = Phe (configuration δ = S, α = S)

C) X = Ile (configuration δ = S, α = R)

D) X = Val (configuration δ = S, α = R)

Fig. 1. Structure of lactam-containing peptides.

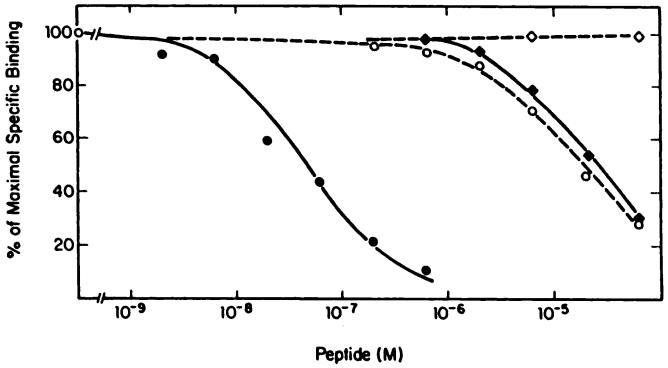


Fig. 2. Inhibition of ^{125}I-BH-SP and ^{125}I-BH-eledoisin binding to rat brain cortex membranes by IA and IB. ^{125}I-BH-eledoisin (*closed symbols*) (0.5 nM) and ^{125}I-BH-SP (*open symbols*) (0.4 nM) binding to rat brain cortex membranes (100 μg of protein) was performed as described in Materials and Methods. IA, ● and ○; IB, ◆ and ◇.

The conformationally constrained analogs, IA, IC, and ID displace ^{125}I-BH-eledoisin from cortex membranes as effectively as does eledoisin itself, while they are more than 2000-fold less effective than substance P in displacing ^{125}I-BH-SP from this same preparation (Fig. 2, Table 1). IB, which differs from IA only in chiralty at the lactam α-carbon, is much less effective in displacing either ligand (Fig. 2). Thus, IA, IC, and ID are selective inhibitors of ^{125}I-BH-eledoisin binding and this selectivity is stereospecific.

IA, IC, and ID are potent agonists in the guinea pig ileum

[2] We tentatively propose this general nomenclature which describes a 2-carbon bridge between the α-carbon of the first residue of a dipeptide to the α-nitrogen (N′) of the second residue of the same dipeptide. For abbreviation of the peptide unit we would propose to place the abbreviation (ANC-2) between the units so bridged. Further, when the α-carbon is that of glycine, chirality would be given to the glycine residue. Thus IA is abbreviated <Glu-Phe-Phe(R)Gly[ANC-2]Leu-Met-NH_2.

TABLE 1

Inhibition of ^{125}I-BH-SP and ^{125}I-BH-eledoisin binding by tachykinin peptides

	IC_{50}[a]		
	^{125}I-BH-SP		^{125}I-BH-eledoisin
	Cortex	Parotid	Cortex
	nM		nM
Substance P	10 ± 5 (7)[b]	13 ± 9 (15)[c]	210 ± 160 (4)[b]
Eledoisin	360 ± 50 (3)[b]	310 ± 130 (2)[c]	31 ± 20 (4)[b]
Substance P methyl ester	20 ± 9 (3)[b]	150 ± 70 (2)[c]	24 ± 5 × 10^3 (3)[b]
<Glu-Phe-Phe-(*R*)Gly[ANC-2]Leu-Met-NH_2 (1A)	16 ± 5 × 10^3 (9)	25 ± 3 × 10^3 (3)	32 ± 21 (11)
<Glu-Phe-Phe-(*S*)Gly[ANC-2]Leu-Met-NH_2 (1B)	>67 × 10^4 (3)	50 ± 21 × 10^4 (2)	30 ± 4 × 10^3 (2)
<Glu-Phe-Ile-(*R*)Gly[ANC-2]Leu-Met-NH_2 (1C)	15 ± 2 × 10^4 (3)	29 ± 2 × 10^4 (2)	170 ± 40 (3)
<Glu-Phe-Val-(*R*)Gly[ANC-2]Leu-Met-NH_2 (1D)	25 ± 13 × 10^4 (3)	42 ± 1 × 10^4 (2)	98 ± 33 (3)
<Glu-Phe-Phe-Gly-Leu-Met-NH_2	420 ± 110 (6)	380 ± 60 (3)	36 ± 25 (5)
<Glu-Phe-Ile-Gly-Leu-Met-NH_2	490 ± 60 (3)	600 ± 90 (3)	14 ± 2 (3)
<Glu-Phe-Val-Gly-Leu-Met-NH_2	2.0 ± 0.2 × 10^3 (3)	2.8 ± 0.9 × 10^3 (3)	19 ± 3 (3)

[a] Mean ± SD; (*n*) = number of determinations.
[b] From Ref. 7.
[c] From Ref. 6.

TABLE 2

Biological potency of tachykinin peptides

	ED_{50}		
	Guinea pig ileum[a]	Salivation[a]	Rat vas deferens[b]
	nM	nmol/100 g body wt	μM
Substance P	0.44 ± 0.08 (2)	0.08 ± 0.03 (7)[c]	13.6 (8.5–19.7)
Eledoisin		0.13 ± 0.09 (2)[c]	0.50 (0.13–1.41)
Substance P methyl ester	1.7 ± 0.6 (2)	0.32 ± 0.14 (3)[c]	> 100
<Glu-Phe-Phe-(*R*)Gly[ANC-2]Leu-Met-NH_2 (1A)	1.2 ± 0.8 (2)	> 40	2.1 (1.6–2.8)
<Glu-Phe-Phe-(*S*)Gly[ANC-2]Leu-Met-NH_2 (1B)	≥ 1000 (2)	> 26	> 100
<Glu-Phe-Ile-(*R*)Gly[ANC-2]Leu-Met-NH_2 (1C)	16.4 (2)	> 40	4.4 (3.4–7.3)
<Glu-Phe-Val (*R*)Gly[ANC-2]Leu-Met-NH_2 (1D)	10.0 (2)	> 40	1.9 (2.4–3.3)
<Glu-Phe-Phe-Gly-Leu-Met-NH_2		0.48 (1)	20.6 (12.0–35.1)
<Glu-Phe-Ile-Gly-Leu-Met-NH_2		0.56 (1)	0.73 (0.51–1.16)
<Glu-Phe-Val-Gly-Leu-Met-NH_2		1.6 (1)	2.9 (2.2–4.2)

[a] Mean ± SD; (*n*) = number of determinations.
[b] Geometric mean of at least three determinations (range).
[c] From Ref. 6.

assay (Table 2). Recent pharmacological data indicates that this tissue contains a mixed population of receptors and may contain both P and E type sites (15–17). In contrast to their activity in the guinea pig ileum, these peptides are at least 500 times less potent than substance P in stimulating salivation in rats (Table 2). The rat vas deferens has been described as an E type tissue (11), and we find that eledoisin is 26-fold more active than substance P in this tissue, while substance P methyl ester is virtually inactive (Table 2). IA, IC, and ID are potent agonists in rat vas deferens (Table 2), although all are less potent than eledoisin (4-, 8-, and 4-fold, respectively). The stereoisomer IB is inactive in all three assays.

It is noteworthy that the substance P fragment, <Glu-Phe-Phe-Gly-Leu-Met-NH_2 as well as the fragments derived from eledoisin, <Glu-Phe-Ile-Gly-Leu-Met-NH_2, and from neurokinin B, <Glu-Phe-Val-Gly-Leu-Met-NH_2, are much less potent in displacing ^{125}I-BH-SP than ^{125}I-BH-eledoisin from cortex membranes (Table 1). This indicates some contribution to substance P binding to cortex by the amino-terminal portion. The three peptides are relatively weak in stimulating salivation, and have only 16%, 14%, and 5% of the activity of substance P, respectively (Table 2). In the rat vas deferens assay, <Glu-Phe-Ile-Gly-Leu-Met-NH_2 and <Gly-Phe-Val-Gly-Leu-Met-NH_2 are potent agonists. However, <Glu-Phe-Phe-Gly-Leu-Met-NH_2 is only 4% and 14% as potent as the eledoisin- and neurokinin B-derived fragments, respectively (Table 2).

IA (1 nM) and substance P methyl ester (1 nM) both produce

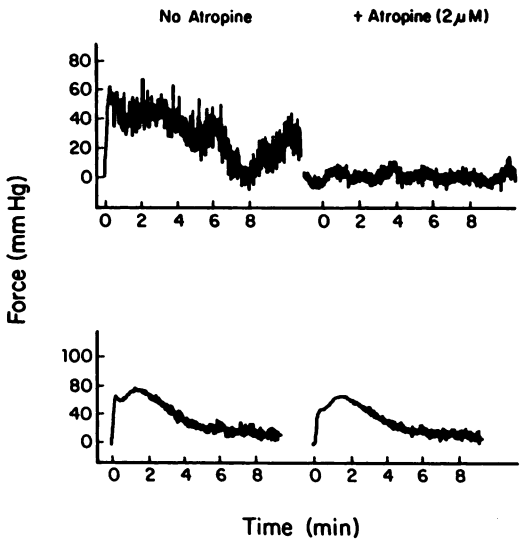


Fig. 3. Differential effect of atropine on the stimulation of guinea pig ileum contraction by IA (*top*) and substance P methyl ester (*lower*). Guinea pig ileum assay was performed as described in Materials and Methods. Atropine was added 10 min prior to addition of peptide.

a strong contraction of guinea pig ileum (Fig. 3). In the presence of atropine (2 μM), the IA-induced contraction is abolished, whereas atropine has no effect on the substance P methyl ester-induced contraction (Fig. 3). Atropine causes a rightward shift

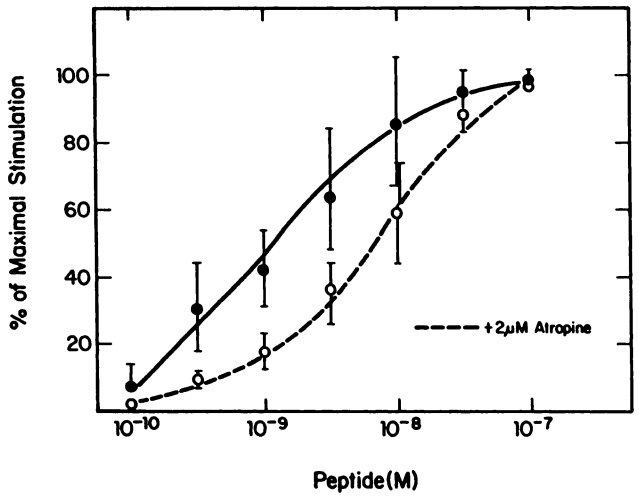


Fig. 4. The stimulation of guinea pig ileum contraction by IA in the presence and absence of atropine. Guinea pig ileum assay was performed as described in the legend to Fig. 3 using the concentrations of IA as shown. Data are the mean ± SD of two experiments. The relative potency of IA in the presence and absence of atropine is different ($p < 0.05$) as determined by analysis of variance using a method for parallel line bioassays (18).

in the dose response curve for IA (Fig. 4). The ED_{50} for IA is shifted from 1.2 nM in the absence of atropine to 6.7 nM in the presence of atropine ($p < 0.05$), while the maximal response of IA is not affected by atropine.

Discussion

The conformationally constrained peptides, IA, IC, and ID, are potent and selective inhibitors of ^{125}I-BH-eledoisin binding and potent agonists in the guinea pig ileum assay. We previously showed that rat parotid cells contain a nearly homogenous population of ^{125}I-BH-SP receptors, since specific binding of ^{125}I-BH-eledoisin is not demonstrable at protein concentrations 5 times higher than those required to demonstrate ^{125}I-BH-SP binding (7). Thus, since these peptides are weak inhibitors of ^{125}I-BH-SP binding, they are very weak stimulators of rat salivation. In contrast, IA, IC, and ID are potent agonists in the rat vas deferens assay. In this tissue, eledoisin is 26 times more potent than substance P and substance P methyl ester is inactive.

The high potency of IA in competing for ^{125}I-BH-eledoisin binding indicates that the lactam constraint is compatible with the receptor-bound conformation at this receptor. In contrast, the reduced displacement of ^{125}I-BH-substance P indicates that a different conformation is recognized at that receptor. Different active conformations at the various opioid receptors have similarly been proposed on the basis of the selectivity of conformationally constrained analogs of enkephalin (19).

Specific speculations about the ^{125}I-BH-eledoisin receptor-bound conformation may now be made. Preliminary NMR[3] studies suggest a preferred conformation of IA in solution (dimethyl sulfoxide) having a turn which does not fit any of the classic definitions for the various types of peptide turns. It also differs from the dimethyl sulfoxide solution conformation proposed for physalaemin (20). The solution conformation of IA results in an internal hydrogen bond involving the carboxy-terminal amide group. The specific contribution of hydrogen bonds to the conformation of small peptides is not clear cut. However, the local changes (steric or electronic) brought about by the methyl ester compared to an amide group may selectively destabilize the eledoisin-preferred conformation and consequently give the observed selectivity of substance P methyl ester.

The peptide backbone of IB would be expected to have a type II′ β-turn by analogy to luteinizing hormone-releasing hormone where various constrained analogs including the *S*-lactam (13) point to the presence of this specific turn type. Therefore, we conclude that the ^{125}I-BH-SP and ^{125}I-BH-eledoisin type receptor-bound conformations do not have type II′ β-turns.

The high potency of IA leads to the prediction that replacement of glycine by D-proline should also give potent analogs. The studies by Piercey *et al.* (21) in fact show D-Pro^{9}SP (6–11) (the D-proline analog which corresponds to IA) as well as a lactam analog similar to IA to be selective.

The principle difference in the carboxyl-terminal sequences of eledoisin, neurokinin B, and substance P is the substitution of the phenylalanine in the latter with the branched chain amino acids isoleucine and valine, respectively. However, our data show that the substitution of the phenylalanine with isoleucine or valine in the hexapeptide fragments and in the conformationally constrained analogs has only a small effect on the potency of the peptides as inhibitors of ^{125}I-BH-eledoisin binding. All of the hexapeptide fragments are weak inhibitors of ^{125}I-BH-SP binding, and even though the isoleucine- and valine-substituted peptides are somewhat less potent, it is clear that the presence of an aromatic or branched chain amino acid at this position in the sequence is not the primary contributor to the selectivity of ^{125}I-BH-eledoisin and ^{125}I-BH-SP for their respective binding sites. We conclude that as yet undetermined amino acid residues in the amino-terminal sequence of substance P strongly contribute to the carboxyl-terminal peptide selectivity and particularly to its potency at the substance P receptor.

The relatively low potency of the hexapeptide fragments as stimulators of salivation correlates well with their ability to inhibit ^{125}I-BH-SP binding to cortex membranes or to parotid cells. In contrast, while all three hexapeptide fragments are potent inhibitors of ^{125}I-BH-eledoisin binding to cortex membranes, only <Glu-Phe-Ile-Gly-Leu-Met-NH_2 and <Glu-Phe-Val-Gly-Leu-Met-NH_2 are potent stimulators of rat vas deferens contraction. It is possible that the poor activity of <Glu-Phe-Phe-Gly-Leu-Met-NH_2 is due to a higher rate of proteolytic degradation of this peptide under the assay conditions. Alternatively, the receptor population(s) of the rat vas deferens may be different than the binding site recognized by ^{125}I-BH-eledoisin in rat cortex membranes, even though eledoisin is much more actice in this tissue than substance P and substance P methyl ester is inactive. The latter possibility is supported by the observation of Buck *et al.* (22) that the relative potencies of selected tachykinins to inhibit the binding of ^{125}I-BH-eledoisin to cortex membranes and to various smooth muscle membrane preparations are different. Also, more recent pharmacological characterization of various smooth muscle preparations using substance P, neurokinins A and B, and nonmammalian tachykinins suggest that there are at least three subtypes of tachykinin receptors (23).

The substance P-induced contraction of guinea pig ileum is not inhibited by atropine. However, Holzer and Lembeck (15)

[3] Unpublished data.

showed that after inducing a maximal longitudinal contraction by high levels of substance P, the contraction was not followed by complete relaxation. This remaining contraction could be enchanced by the cholinesterase inhibitor physostigmine and was decreased by the anticholinergic tropicamide. Subsequently, Yau and Youther (16) were able to directly demonstrate release of acetylcholine from the myenteric plexus by substance P. Recently, Fosbraey *et al.* (17) showed that eledoisin was 26 times more potent than substance P in eliciting acetylcholine release. Our data indicate that the IA-induced contraction of guinea pig ileum is largely dependent on the release of acetylcholine. Thus, IA may have a higher affinity for the tachykinin receptors in the myenteric plexus which are responsible for acetylcholine release than for the tachykinin receptors in the ileal smooth muscle.

This demonstration of distinct tachykinin receptor populations in the myenteric plexus and the smooth muscle cells of the guinea pig ileum indicates the utility of using substance P methyl ester and IA as receptor-specific probes. In addition, the selectivity of these peptides provide evidence of different conformational requirements for ^{125}I-BH-SP and ^{125}I-BH-eledoisin receptor binding. It should now be possible to design additional conformationally restricted analogs which will more fully define the ^{125}I-BH-eledoisin receptor-bound conformation.

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