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Highly selective agonists for substance P receptor subtypes

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The existence of a third tachykinin receptor (SP-N) in the mammalian nervous sytem was demonstrated by development of highly selective agonists. Systematic N-methylation of individual peptide bonds in the C-terminal hexapeptide of substance P gave rise to agonists which specifically act on different receptor subtypes. The most selective analog of this series, succinyl-[Asp^6,Me-Phe^8]SP$_{6-11}$, elicits half-maximal contraction of the guinea pig ileum through the neuronal SP-N receptor at a concentration of 0.5 nM. At least 60 000-fold higher concentrations of this peptide are required to stimulate the other two tachykinin receptors (SP-P and SP-E). The action of selective SP-N agonists in the guinea pig ileum is antagonized by opioid peptides, suggesting a functional counteraction between opiate and SP-N receptors. These results indicate that the tachykinin receptors are distinct entities which may mediate different physiological functions.

Key words: tachykinins/neurokinin B/substance P/opioid peptides/guinea pig ileum

Introduction

Substance P, a neuropeptide of 11 amino acids, is a neurotransmitter or neuromodulator in the central and peripheral nervous system. This peptide has been implicated in a variety of physiological functions, including transmission of pain stimuli, exocrine gland secretion, intestinal motility, vasodilation and the neuronally mediated inflammatory skin reaction (Nicoll *et al.*, 1980; Pernow, 1983; Bartho and Holzer, 1985). Recently, two additional mammalian substance P-related peptides, neurokinin A (also known as substance K) and neurokinin B (also known as neuromedin K) were identified (Kimura *et al.*, 1983; Kangawa *et al.*, 1983). They share with substance P the common C-terminal sequence Phe-X-Gly-Leu-Met-NH$_2$ which characterizes the tachykinin family of peptides (Erspamer, 1981). It has been suggested that the actions of the different tachykinins are mediated by multiple receptors, two of which have been classified as SP-P and SP-E subtypes (Lee *et al.*, 1982; Watson *et al.*, 1983). SP-E receptors were shown to preferentially interact with neurokinin A (Nawa *et al.*, 1984; Holzer-Petsche *et al.*, 1985; Osakada *et al.*, 1986). We have recently proposed the existence of a third receptor subtype (SP-N) whose preferred ligand is neurokinin B (Laufer *et al.*, 1985). A neurokinin B receptor has also been identified by binding studies in the mammalian central nervous sytem (Torrens *et al.*, 1984; Buck *et al.*, 1984).

The notion that a single neurotransmitter can act on more than one type of receptor is well established for the small non-peptide neurotransmitters (Snyder, 1984). This is not clear, however, for larger neuropeptides and peptide hormones, probably due to the fact that development of selective agonists and antagonists for a particular receptor subtype is much more difficult with such complex structures. Since a single neurotransmitter acting on two different receptors can elicit a variety of different and sometimes opposed effects in complex tissues and in whole animals, selective receptor ligands are highly desirable. Selective agonists are of great importance not only for basic studies directed at analysis of the mechanism by which the peptide produces its effect, but also for therapeutic applications that require selective activation or inhibition of a subset of cells bearing a particular receptor type (Snyder, 1984).

Most neuropeptides are small linear molecules that have considerable conformational freedom. The rationale for development of selective agonists is based on the assumption that the naturally occurring transmitters can exist in several different and interchangeable conformations, each selected for or induced by a particular receptor subtype (Hruby, 1985). From this consideration it follows that conformationally restricted analogs should reveal selectivity towards specific receptor subtypes. This approach was successfully applied to the design of cyclic enkephalin and somatostatin analogs that selectively act on subclasses of opiate and somatostatin receptors, respectively (Veber *et al.*, 1981; DiMaio *et al.*, 1982; Mosberg *et al.*, 1983; Hruby, 1985; Tran *et al.*, 1985). On the other hand, cyclic analogs of substance P in a variety of constructions (Chassaing *et al.*, 1984; Sandberg, 1985; Darman *et al.*, 1985; Theodoropoulos *et al.*, 1985) were found to be biologically inactive, apparently because rigidification via cyclization resulted in multiple changes in the conformation of the substance P molecule. In an attempt to overcome this problem, we have chosen to introduce limited conformational constraints by N-alkylation (Manavalan and Momany, 1980) of a single peptide bond in the C-terminal hexapeptide of substance P. In addition to its conformational effect, the replacement of an amide bond hydrogen by an alkyl group could abolish specific hydrogen bonds involved in ligand–receptor interactions. N-Methylation of peptide bonds has so far been used to confer metabolic stability on substance P analogs (Laufer *et al.*, 1981; Sandberg *et al.*, 1981; Wormser *et al.* 1984). It is now shown that such a modification results in substantial loss of biological activity towards one receptor subtype, while the activity on the other receptor remains unchanged. Furthermore, N-alkylation of different peptide bonds gave rise to analogs with inverse spectra of selectivity.

Results

Two tachykinin receptors are recognized in the guinea pig ileum: a muscular receptor (SP-P) which elicits contraction by direct action on the muscle cells, and a neuronal receptor (SP-N) which elicits contraction indirectly through stimulation of acetylcholine release from cholinergic neurones (Laufer *et al.*, 1985). The released acetylcholine in turn evokes contraction via

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muscarinic acetylcholine receptors located on the muscle cells. It has been proposed that the guinea pig ileum may also contain SP-E receptors (Lee *et al.*, 1982; Watson *et al.*, 1983), although these might have been confused with SP-N sites (Laufer *et al.*, 1985). The hexapeptide [pyroglutamyl^6]substance P (6−11) hexapeptide ([pGlu^6]SP_{6-11}) is a potent agonist for both SP-P and SP-N receptors in the guinea pig ileum (see Figure 1). In an effort to prepare ligands with improved selectivity for different tachykinin receptors, analogs of [pGlu^6]SP_{6-11} that contain one N-alkylated peptide bond, were synthesized. All of these peptides proved to be full agonists for SP-P and SP-N receptors. As shown in Figure 1, N-methylation of Leu^{10} does not significantly affect activity on either receptor subtype and thus does not improve selectivity. Although N-methylation of Phe^7 increases selectivity for the SP-P receptor, the modified peptide [pGlu^6,Me-Phe^7]SP_{6-11} is a weak SP-P agonist possessing only 5% of the activity of [pGlu^6]SP_{6-11}. In contrast, substitution of Gly^9 by its N-methylated derivative sarcosine did not impair SP-P potency while it slightly reduced activity on the SP-N receptor. This result prompted us to replace Gly^9 by a proline residue since the incorporation of the N-alkyl group as part of a cyclic structure is expected to result in even greater conformational restriction. As previously reported (Laufer *et al.*, 1986a), [pGlu^6,Pro^9]SP_{6-11} is a potent and highly selective SP-P agonist. Substitution of Gly^9 by Pro^9 had almost no effect on potency for SP-P receptor, but reduced activity on the SP-N receptor by two orders of magnitude. This compound was designated 'Septide' (*Se*lective SP-*P* receptor pep*tide*).

Most remarkably, an opposite effect on receptor selectivity was obtained by N-methylation of the Phe^8 residue. This modification resulted in a 10-fold increase in agonist potency for the SP-N receptor and a concomitant 100-fold decrease in the potency of the analog for the SP-P receptor. Consequently, [pGlu^6,Me-Phe^8]SP_{6-11} is a highly potent and selective SP-N agonist. N-methylation of both Phe^8 and Gly^9, each of which produces opposite effects on selectivity, yields an analog, [pGlu^5,Me-Phe^8,Sar^9]SP_{5-11}, of low potency and little receptor selectivity. This peptide has been previously reported by Sandberg *et al.* (1981) as a metabolically stable analog.

In agreement with our previous observation that SP-N receptor activity could be modulated by opioid peptides (Laufer *et al.*, 1985), it was found that the contractile action of [pGlu^6,Me-Phe^8]SP_{6-11} in the guinea pig ileum is inhibited by [D-Ala^2,Met^5]enkephalinamide in a naloxone-sensitive manner (Figure 2). Similar results were obtained in the presence of 20 nM of dynorphin (1−13) (data not shown).

The high potency of [pGlu^6,Me-Phe^8]SP_{6-11} for the neuronal neurokinin B receptor (SP-N) in guinea pig ileum was confirmed by direct measurement of [^{3}H]acetycholine release from prelabeled strips of guinea pig ileum longitudinal muscle-myenteric plexus. As shown in Figure 3, [pGlu^6,Me-Phe^8]SP_{6-11} has an EC_{50} value of 1 nM in causing [^{3}H]acetylcholine release from enteric neurones, and is thus equipotent with neurokinin B and about 50 times more potent than substance P and neurokinin A in this system.

The selective action of [pGlu^6,Me-Phe^8]SP_{6-11} and Septide on the neuronal and muscular tachykinin receptors of the guinea pig ileum is illustrated in Figure 4. As expected for a selective SP-P agonist, the contractile effect of Septide (EC_{50} = 2 nM) is not affected by the muscarinic blocker atropine but it is reduced 600-fold following inactivation of the SP-P receptor by desensitization with the selective SP-P agonist substance P methyl ester

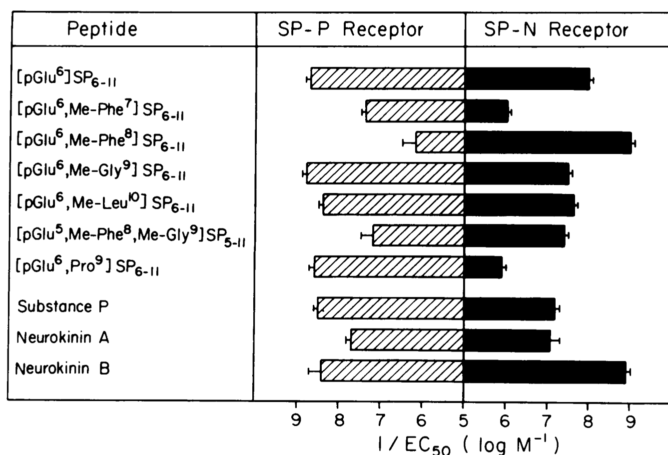

Fig. 1. N-Alkylation of different peptide bonds in [pGlu^6]SP_{6-11} increases agonist selectivity for either the muscular SP-P or for the neuronal SP-N receptor in guinea pig ileum. Selective assays of SP-P and SP-N receptors were performed as described in Materials and methods. The data are EC_{50} values ± SEM from 3−10 experiments.

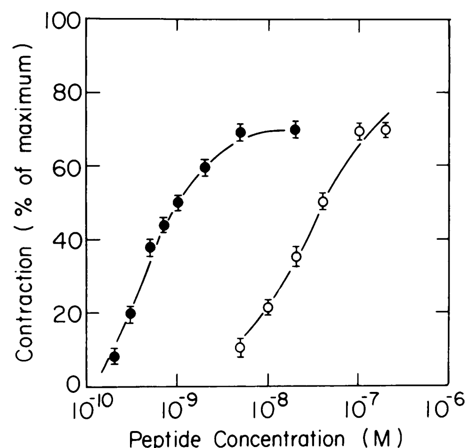

Fig. 2. [D-Ala^2,Met^5]Enkephalinamide inhibits the contractile action of [pGlu^6,Me-Phe^8]SP_{6-11} in the guinea pig ileum. A dose−response curve of [pGlu^6,Me-Phe^8]SP_{6-11} was established in the absence (●) or in the presence (○) of 1 μM of [D-Ala^2,Met^5]enkephalinamide. The effect of the opioid peptide was prevented by 1 μM naloxone (data not shown).

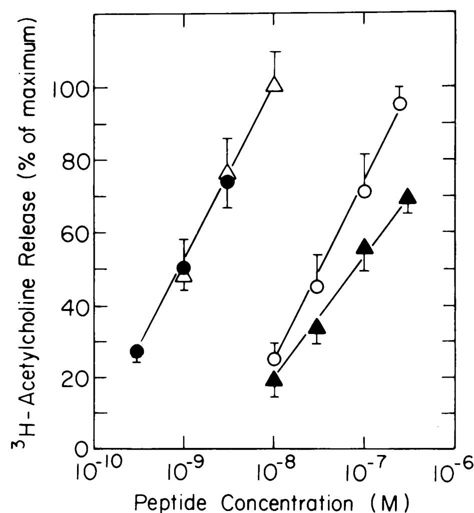

Fig. 3. Potencies of substance P-related peptides in stimulating [^{3}H]acetylcholine release in guinea pig ileum. △ neurokinin B; ● [pGlu^6,Me-Phe^8]SP_{6-11}; ○ substance P; ▲ neurokinin A.

(Laufer *et al.*, 1986a). On the other hand, the dose–response curve of the selective SP-N agonist [pGlu^6,Me-Phe^8]SP$_{6-11}$ is shifted ~1000-fold to the right upon blockage by atropine of the neurogenic component of contraction. This finding reflects the very low potency of this peptide in stimulating the muscular SP-P receptor. This result is corroborated by the inability of high concentrations of [pGlu^6,Me-Phe^8]SP$_{6-11}$ to stimulate K^+ and α-amylase release from rat parotid slices or to cause hypotension in rabbits (data not shown), two effects mediated by SP-P receptors (Pernow, 1983). The selectivity of this peptide for the SP-N receptor is also evident from the finding that its action in the guinea pig ileum is not affected by inactivation of the SP-P receptor (Figure 4).

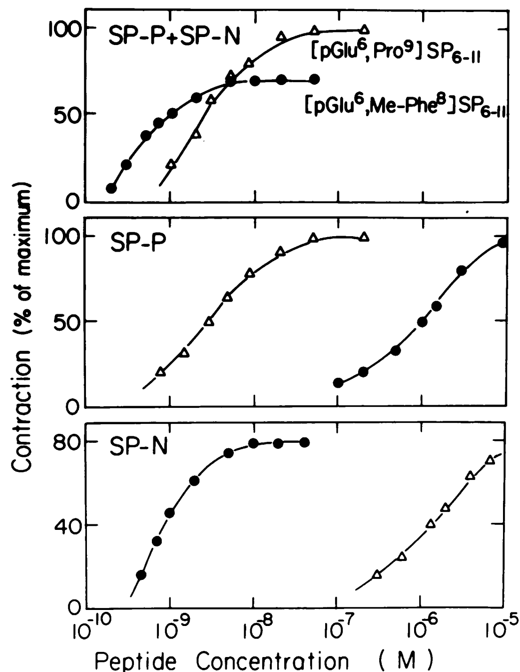

Fig. 4. Selective agonists discriminate between SP-P and SP-N receptors in guinea pig ileum. Dose–response curves of [pGlu^6,Me-Phe^8]SP$_{6-11}$ (●) and [pGlu^6,Pro^9]SP$_{6-11}$ (△) were established in the guinea pig ileum. In the absence of any addition, the measured response is the resultant of peptide action on both SP-P and SP-N receptors. Selective assays of the SP-P and SP-N receptors were performed in the presence of atropine and substance P methyl ester, respectively, as described in Materials and methods. Note that the maximal contraction obtained by stimulation of the SP-N receptor is somewhat lower than that elicited by stimulation of the SP-P receptor. All tachykinins produce the same maximal contraction when acting on the SP-N receptor.

Table I. Selective agonists discriminate between three tachykinin receptor subtypes.

Peptide	EC$_{50}$ (nM) Assay system:	Guinea pig ileum	Guinea pig ileum	Hamster bladder	Rat vas deferens
	Receptor subtype:	SP-P	SP-N	SP-E	SP-E
Senktide		35 000	0.5	30 000	>200 000[b]
Septide[a]		2.5	1300	33 000	>40 000[b]
Substance P		3	59	2500	20 000
Neurokinin B		4	1.3	50	400

Contraction of hamster urinary bladder and potentiation of electrically induced contraction of rat vas deferens were determined by established procedures (Watson *et al.*, 1983). Results are mean values from at least three experiments (standard error <20%)

[a]Data are from Laufer *et al.* (1986a).

[b]No effect at the indicated concentration.

Further improvement in the selectivity for the SP-N receptor was obtained as the result of an effort to increase the water solubility of [pGlu^6,Me-Phe^8]SP$_{6-11}$. While introduction of a positive charge by incorporation of arginine at position 6 had a deleterious effect on potency (data not shown) introduction of negative charge resulted in greatly increased selectivity for the SP-N receptor. The water-soluble analog succinyl-[Asp^6,Me-Phe^8]SP$_{6-11}$ is equipotent to [pGlu^6,Me-Phe^8]SP$_{6-11}$ in stimulating the SP-N receptor (EC$_{50}$ = 0.5 nM) but considerably less potent than the parent compound in its action on the SP-P receptor (EC$_{50}$ = 35 μM). We have designated this highly selective SP-N agonist, which has an SP-N/SP-P potency ratio of 70 000, 'Senktide' (*Se*lective *n*euro*k*inin B receptor pep*tide*).

Selective agonists effectively discriminate between three tachykinin receptors. Indeed, Senktide elicits half-maximal contraction of the hamster urinary bladder (SP-E system; Watson *et al.*, 1983), only at a concentration 60 000 times higher than the EC$_{50}$ value of this peptide for the SP-N receptor in guinea pig ileum. Furthermore, Senktide is devoid of activity in another SP-E system, the rat vas deferens, even when tested at a concentration 400 000 times higher than its EC$_{50}$ value for the SP-N receptor (Table I). It is noteworthy that Senktide is much less potent than substance P or [pGlu^6]SP$_{6-11}$, which themselves are poor SP-E agonists (Watson *et al.*, 1983; Laufer *et al.*, 1986a).

Discussion

In the present study, conformationally restricted analogs of substance P were developed that are highly selective agonists of specific tachykinin receptor subtypes. The selectivity of N-alkylated substance P analogs for either the SP-P or SP-N receptor is due mainly to loss of their ability to activate one of the two receptors. It remains to be established whether this is due to their inability to adopt a specific conformation required for activation of one of the receptors or whether removal of the amide hydrogen from the peptide bond abolishes an essential hydrogen bond interaction with that receptor.

Recently, other substance P analogs have been reported to possess some selectivity for tachykinin receptor subtypes. The hexapeptide analogs [Pro^9]SP$_{6-11}$ and [D-Pro^9]SP$_{6-11}$ seem to discriminate between putative SP-P receptor subtypes designated 'SP$_1$' and 'SP$_2$'. It should be pointed out that these peptides are 100–1000 times less potent than [pGlu^6,Pro^9]SP$_{6-11}$ in the guinea pig ileum (Piercey *et al.*, 1985). Their potencies on the two other tachykinin receptor types (SP-N and SP-E) have not as yet been reported. Cascieri *et al.* (1986) have developed a conformationally restricted substance P analog that preferentially interacts with eledoisin binding sites rather than with substance P binding sites in rat brain. This compound also potently stimulates SP-E receptors of rat vas deferens. Since eledoisin binding sites have been shown to consist of a heterogeneous population of sites, part of which seem to represent neurokinin B receptors (Buck *et al.*, 1984; Cascieri *et al.*, 1985), it remains to be established whether the analog reported by Cascieri and co-workers can discriminate between neurokinin A (SP-E) and neurokinin B (SP-N) receptors. Moreover, the small (6-fold) reduction in the potency of this compound in guinea pig ileum, following blockage of the cholinergic component of contraction, contrasts with 1000-fold and 70 000-fold shifts in the dose–response curves of [pGlu^6,Me-Phe^8]SP$_{6-11}$ and Senktide, respectively. These results show that the latter peptides are the most selective SP-N receptor ligands developed to date. Senktide clearly discriminates between SP-N,

SP-P and SP-E receptors, with selectivity (potency) ratios >60 000. Because of its high selectivity for SP-N receptors, a radiolabeled analog of Senktide could be used to identify a single population of binding sites in rat brain for which Senktide has an affinity constant close to its EC_{50} value in the guinea pig ileum. The pharmacological specificity of this binding site is very similar to that of the ileal SP-N receptor. In particular, neurokinin B was found to be the preferred ligand for the Senktide binding site in rat brain, showing that Senktide selectively interacts with a tachykinin SP-N receptor type in both the central and peripheral nervous sytem (Laufer *et al.*, 1986b).

Selective tachykinin receptor ligands are important tools for further studies on the role of tachykinins in specific physiological functions. The functional interaction between naturally occuring tachyinins and opioid peptides in the guinea pig ileum could so far be observed only after pharmacological inactivation of the muscular tachykinin receptors (Holzer *et al.*, 1983; Laufer *et al.*, 1985). In contrast, a direct inhibition by opioid peptides of tachykinin receptor-mediated contraction can be demonstrated using SP-N-selective tachykinin agonists. The mechanism of this inhibition is at present unknown; further studies are necessary to determine whether the inhibitory effect of opioid peptides is due to a post-synaptic blockade of cholinergic cell firing or to inhibition of acetylcholine release from nerve terminals (North and Williams, 1983). It can be expected that the selective agonists described in this work will be found useful in probing the functional counteraction between opiate and SP-N receptors.

Materials and methods

Peptides

Peptides were prepared by conventional methods in solution or by solid phase techniques. Tachykinins were obtained from commercial sources (Laufer *et al.*, 1985).

Smooth muscle contraction assay

Segments of smooth muscle preparations were suspended in a 10 ml organ bath containing Tyrode's solution (composition in mM: NaCl, 118; KCl, 4.7; $CaCl_2$, 1.8; $MgCl_2$, 0.5; NaH_2PO_4, 1.0; $NaHCO_3$, 25; and glucose 10), gassed with a 95% O_2/5% CO_2 mixture and maintained at 34°C. Contractions were recorded isotonically (Laufer *et al.*, 1985).

Selective assay of the muscular SP-P receptor in guinea pig ileum was performed in the presence of 0.3 μM atropine, a muscarinic blocker which prevents the action of neuronally released acetylcholine and thereby eliminates the neurogenic component of the contractile response. Selective assay of the neuronal SP-N receptor was performed following inactivation of the muscular receptor by desensitization with the selective SP-P agonist substance P methyl ester (0.1 μM, 2 min pre-treatment of the tissue) as described before (Laufer *et al.*, 1985). In the presence of substance P methyl ester, the tachykinin-induced contraction is mediated by SP-N receptors, leading to acetylcholine release from enteric neurons (Laufer *et al.*, 1985). All test peptides were full agonists in a given receptor assay. EC_{50} is the concentration of agonist needed to cause half-maximal contraction.

Determination of tachykinin-induced [^{3}H]acetylcholine release from guinea pig ileum strips

Strips of guinea pig ileum longitudinal muscle with attached myenteric plexus were prepared by the method of Paton and Zar (1968). The strips were incubated for 1 h with Tyrode's solution containing 1 μM of [^{3}H]choline (15 Ci/mmol; Amersham) in a shaking water bath at 37°C. During the entire experiment the Tyrode solution was continually gassed with a mixture of 95% O_2/5% CO_2. The strips were then thoroughly washed and cut into pieces of 2−3 mm length that were distributed to individual systems. These consisted of small baskets sealed with nylon mesh at one end and immersed in plastic wells containing 1 ml of medium. The baskets were transferred every 5 min to wells containing fresh Tyrode's solution supplemented with 20 μM hemicholinium-3 and 0.05% BSA. After 1 h, the tachykinin agonist to be tested was added to the medium as a dimethylsulfoxide (DMSO) solution. The final concentration of DMSO was 1% (v/v), at which dose the solvent did not cause release of radioactivity by itself. The tachykinin-induced release was calcium-dependent (data not known) and has been shown to represent [^{3}H]acetylcholine (Vizi and Bartho, 1985). Evoked release was defined as the ratio of net radioactivity released during 5 min stimulation over the basal release in the absence of agonist. Results were expressed relative to the maximal effect evoked by a supersaturating concentration (10 μM) of [pGlu^6,Me-Phe^8]SP_{6-11}. All agonists tested elicted the same maximal response.

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