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NK-3 receptors mediate enhancement of substance P release from capsaicin-sensitive spinal cord afferent terminals

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1 The effects of NK-3 receptor agonists on the release of substance P-immunoreactivity (SP-LI) have been investigated using superfused rat spinal cord synaptosomes.

2 The Ca^{2+}-dependent overflow of SP-LI evoked by 35 mM KCl was concentration-dependently enhanced by senktide (EC_{50} = 52 nM; maximal effect = 70%) or [MePhe^7]NKB (EC_{50} = 5.5 nM; maximal effect = 125%), both selective agonists at receptors of the NK-3 type.

3 The potentiation of the SP-LI overflow elicited by 100 nM senktide or [MePhe^7]NKB was prevented by the NK-3 receptor antagonist (+)-SR142801. The antagonist halved, at 10 nM, and almost abolished, at 100 nM, the effect of both agonists. The effect of senktide or [MePhe^7]NKB was insensitive to antagonists at NK-1 or NK-2 receptors.

4 Capsaicin (0.1–1 μM) stimulated SP-LI release in a concentration-dependent manner from spinal cord synaptosomes. The SP-LI overflow elicited by 1 μM capsaicin was completely dependent on external Ca^{2+}. Senktide could not affect the capsaicin-evoked release of SP-LI.

5 Senktide failed to potentiate the K^+-evoked overflow of SP-LI from synaptosomes previously exposed for 15 min in superfusion to capsaicin.

6 The results show that release-enhancing NK-3 receptors are located on axon terminals of capsaicin-sensitive primary afferent neurones in the spinal cord. Antagonists at NK-3 receptors might help controlling pain transmission.

Keywords: NK-3 receptor; substance P release; capsaicin; spinal cord synaptosomes

Introduction

The role of the neuropeptides substance P (SP) in spinal mechanisms of pain transmission has been the object of innumerable studies. A major source of neuronal SP is represented by the capsaicin-sensitive primary afferent neurones located in dorsal root ganglia, although SP also exists in intrinsic dorsal horn neurones. The release of SP in the spinal cord is increased during nociception to activate receptors of the neurokinin-1 (NK-1) type. Accordingly, non-peptide NK-1 receptor antagonists have been reported to exert analgesic effects in various tests (Yamamoto & Yaksh, 1991; Garret *et al*., 1991; Birch *et al*., 1992; Boyce *et al*., 1992; Maggi *et al*., 1993 for review).

The primary afferent neurones of the dorsal root ganglia also produce neurokinin A (NKA), but not neurokinin B (NKB), a tachykinin that, in spinal cord, is mainly contained in intrinsic neurones (Too & Maggio, 1991) and is thought to activate preferentially receptors of the NK-3 type (see Maggi *et al*., 1993, for review).

Differently from SP, NKB has been attributed anti-nociceptive properties mediated through NK-3 receptors (Couture *et al*., 1993). A calcium-dependent release of NKB in response to depolarizing stimuli has been reported to occur in rat spinal cord preparations (Lindefors *et al*., 1985). Thus the possibility exists that endogenously released NKB modulates SP release onto nociception-specific projection neurones through presynaptic receptors localized on primary afferent terminals.

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We here investigated the effects of NK-3 receptor agonists on the release of SP-like immunoreactivity (SP-LI) from spinal cord synaptosomes and show that activation of NK-3 receptors can enhance SP-LI release from capsaicin-sensitive SP-releasing terminals.

Methods

Animals

Adult male Sprague-Dawley rats (200–250 g) were used. Animals were housed at constant temperature (22 ± 1°C) and relative humidity (50%) under a regular light-dark schedule (light: 07.00 h to 19.00 h). Food and water were freely available.

Preparation of synaptosomes

Rats were killed by decapitation and the spinal cords rapidly removed. Crude synaptosomes were prepared as previously described (Raiteri *et al*., 1984) with some modifications. Briefly, the tissue was homogenized in 40 vol of 0.32 M sucrose, buffered at pH 7.4 with phosphate, using a glass-teflon tissue grinder. The homogenate was first centrifuged at 1000 *g* for 5 min; synaptosomes were isolated from the supernatant by centrifugation at 12,000 *g* for 20 min. All the preceding procedures were performed at 0–4°C. The synaptosomal pellet was then resuspended in a physiological medium having the following composition (mM): NaCl 125; KCl 3; $MgSO_4$ 1.2; $CaCl_2$ 1.2; NaH_2PO_4 1.0; $NaHCO_3$ 22; glucose 10 (aeration with 95% O_2 and 5% CO_2 at 37°C); pH 7.2–7.4. Protein was

measured according to Bradford (1976), using bovine serum albumin as a standard.

Release experiments

Identical aliquots of the synaptosomal suspension (ranging between 0.3–0.6 mg of protein in the different experiments) were layered on microporous filters at the bottom of a set of parallel superfusion chambers maintained at 37°C (Raiteri *et al*., 1974). Superfusion was then started at a rate of 0.5 ml min^{-1} with standard medium supplemented with 0.1% Polypep® and aerated with 95% O_2 and 5% CO_2. After 36 min, to equilibrate the system, fractions were collected according to the following scheme: two 3-min samples (basal release) before and after one 6-min sample (evoked release). A 90 s period of depolarization was applied after the first fraction had been collected. Depolarization of synaptosomes was performed with 15–50 mM KCl (NaCl substituting for an equimolar concentration of KCl) or 0.1–1 μM capsaicin. Senktide or [MePhe^7]NKB was added to the superfusion medium concomitantly with the depolarizing stimulus. (S)-(N)-(1-(3-(1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl) propyl)-4-phenyl piperidin-4-yl)-N-methyl acetamide [(+)- SR 142801], 2-[1-imino-2-(2-methoxyphenyl)ethyl]-7,7-diphenyl-4-perhydroisoindolone (3aR, 7aR) (RP 67580) or [Asn(β-D-GlcNAc)-Asp-Trp-Phe-Dap-Leu]c(2β-5β) (MEN 11420) was introduced 8 min before agonists. When appropriate Ca^{2+}-free medium was introduced after 20 min of superfusion. In some experiments synaptosomes were exposed in superfusion to 10 μM capsaicin for 15 min starting at $t=10$ min of superfusion. Superfusate fractions were collected into vials containing HCl (final concentration 0.1 M).

Radioimmunoassay

Fractions were concentrated on Sep-Pak C_{18} reverse phase silica gel cartridges (Millipore, Milan, Italy) and the eluates were freeze dried before assay. The freeze-dried samples were reconstituted in phosphate buffer (50 mM; pH 7.4) and radioimmunoassayed using an antiserum towards the N-terminus of SP and [^{125}I]-SP as tracer (Lee *et al*., 1980). The assay sensitivity was 4 fmol $tube^{-1}$.

Calculation

The amount of SP released in each fraction was expressed as fmoles mg $protein^{-1}$. The depolarization-evoked overflow was estimated by subtracting the peptide content in the basal release from the release evoked in the 6-min fraction collected during and after the depolarization period.

The drug effects were evaluated as the ratio of the depolarization-evoked overflow calculated in the presence of the drugs vs that calculated under control conditions and expressed as % of potentiation. Appropriate controls with antagonists were always run in parallel. Two-tailed Student *t*-test was used for statistical comparison of two means.

Chemicals

[^{125}I]-Substance P was obtained from Amersham Radiochemical Centre (Buckinghamshire, U.K.); senktide and substance P from RBI (Natick, MA, U.S.A.); [MePhe^7]NKB from Novabiochem (Laufelfingen, Switzerland), capsaicin and Polypep® from Sigma Chemical (St. Louis, MO, U.S.A.). The following drugs were gifts: (+)-SR 142801 (Sanofi Recherche, Montpellier, France), RP 67580 (Rhone Poulenc, Vitry sur Seine, France) and MEN 11420 (Menarini, Firenze, Italy). The authors thank Dr P.C. Emson (Babraham, U.K.) for providing the SP antiserum.

Results

Spinal cord synaptosomes were exposed in superfusion to varying concentrations of KCl (15–50 mM). As illustrated in Figure 1, SP-LI was released in amounts directly related to the external K^+ concentration. The overflows of SP-LI evoked by 25 or 35 mM KCl were almost completely dependent on the presence of Ca^{2+} ions in the superfusion medium. At 50 mM KCl, a consistent portion of the SP-LI overflow occurred independently of external Ca^{2+}. All the subsequent experiments with high-K^+ were performed by depolarizing synaptosomes with 35 mM KCl.

Addition to the superfusion medium of senktide (1 nM–1 μM) or [MePhe^7]NKB (0.1 nM–1 μM), two selective NK-3 receptor agonists, produced concentration-dependent potentiations of the K^+-evoked SP-LI overflow (Figure 2). The maximal effect of senktide (~70%) was significantly lower than that of [MePhe^7]NKB (~125%). Moreover senktide appeared less potent ($EC_{50}=52$ nM) than [MePhe^7]NKB ($EC_{50}=5.5$ nM). Senktide or [MePhe^7]NKB had no effect on the basal SP-LI release (not shown).

The potentiation of the K^+-evoked SP-LI release elicited by the two agonists could be prevented by (+)-SR 142801, a selective non-peptide NK-3 receptor antagonist (Emonds-Alt *et al*., 1995; Beaujouan *et al*., 1997). The effects of senktide and [MePhe^7]NKB, added at 100 nM, were about halved by 10 nM (+)-SR 142801 and almost abolished by 100 nM of the

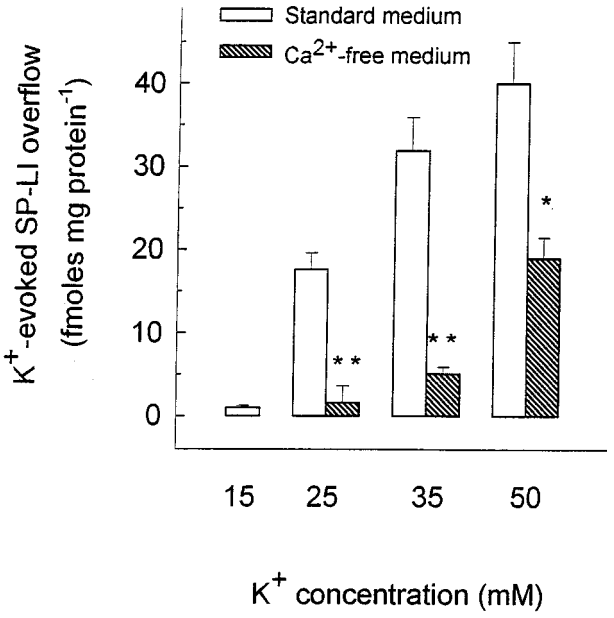

Figure 1 SP-LI overflow (fmoles mg $protein^{-1}$) evoked by varying [K^+] from rat spinal cord synaptosomes and its Ca^{2+} dependence. A 90 s pulse of high K^+ was applied after 39 min of superfusion. When appropriate, Ca^{2+}-free medium was introduced 19 min before high-K^+. Fractions were collected, concentrated on C_{18} Sep-Pak cartridges, freeze dried and radioimmunoassayed. See Methods for other technical details. Basal release (SP-LI content in the 3-min fractions respectively preceding and following the 6-min fraction collected during and after the depolarization pulse) amounted to 22 ± 4 ($n=18$) fmoles mg $protein^{-1}$. Data are means±s.e.mean of 3–7 separate experiments run in triplicate. $^{**}P<0.001$; $^{*}P<0.01$ (two tailed Student's *t*-test).

antagonist (Figure 3). Neither RP 67580 (100 nM), selective NK-1 receptor antagonist, nor MEN 11420 (100 nM), selective NK-2 receptor antagonist, inhibited the effects of 100 nM senktide or [MePhe^7]NKB on the SP-LI evoked overflow (Table 1). The tachykinin receptor antagonists tested had no effect, on their own, on the basal or K^+-evoked SP-LI release (not shown).

In order to shed light on the neuronal site of action of the NK-3 receptor agonists, experiments were performed with capsaicin. As shown in Figure 4, addition of capsaicin (0.1–1 μM) to the superfusion medium elicited concentration-dependent release of SP-LI from spinal cord synaptosomes. The capsaicin (1 μM)-evoked SP-LI overflow was completely Ca^{2+}-dependent (Figure 4).

We first tested senktide on the effect of capsaicin used as the stimulus to evoke SP-LI release. Table 2 shows that senktide, added at 100 or 1000 nM, was unable to increase significantly the overflow of SP-LI provoked by different concentrations (0.1 – 1 μM) of capsaicin.

In another group of experiments synaptosomes were pretreated with capsaicin (10 μM, for 15 min, in superfusion), washed for 15 min with physiological medium, then exposed to senktide during K^+ depolarization. Figure 5 shows that 100 nM senktide failed to potentiate the K^+-evoked SP-LI overflow in synaptosomes which had been pretreated with capsaicin. The Figure also shows that capsaicin pretreatment did not modify significantly the 35 mM K^+-evoked SP-LI release, but completely prevented the release of SP-LI caused by 1 μM capsaicin.

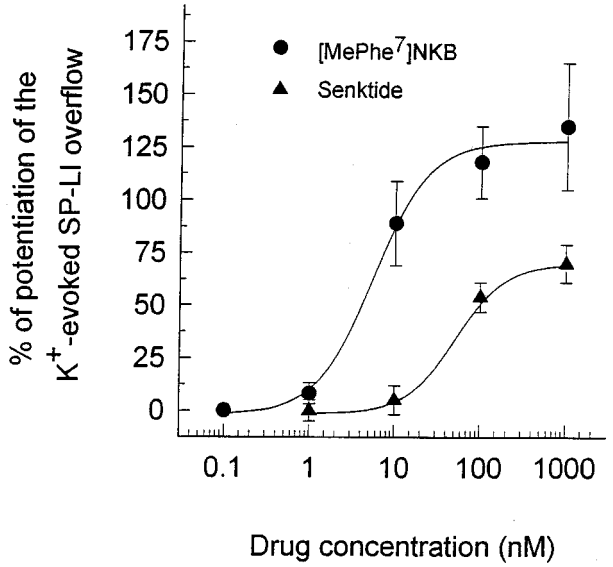

Figure 2 Effects of [MePhe^7]NKB and senktide on the K^+ (35 mM)-evoked SP-LI overflow from rat spinal cord synaptosomes. The agonists were added to the superfusion medium concomitantly with the depolarizing stimulus. See Methods for calculations and other technical details. Data are means ± s.e.mean of three to eight separate experiments run in triplicate.

Table 1 Lack of effect of NK-1 or NK-2 receptor antagonists on the potentiation of SP-LI overflow induced by senktide (100 nM) or [MePhe^7]NKB (100 nM) in rat spinal cord synaptosomes

Drugs	*% of potentiation of the K^+-evoked SP-LI overflow*	n
Senktide	43 ± 7	4
Senktide + RP 67580 (100 nM)	52 ± 8	3
Senktide + MEN 11420 (100 nM)	37 ± 10	3
[MePhe^7]NKB	109 ± 11	3
[MePhe^7]NKB + RP 67580 (100 nM)	130 ± 10	3
[MePhe^7]NKB + MEN 11420 (100 nM)	137 ± 15	3

See Methods for calculations and technical details. Values represent means ± s.e.mean of data obtained in *n* separate experiments run in triplicate.

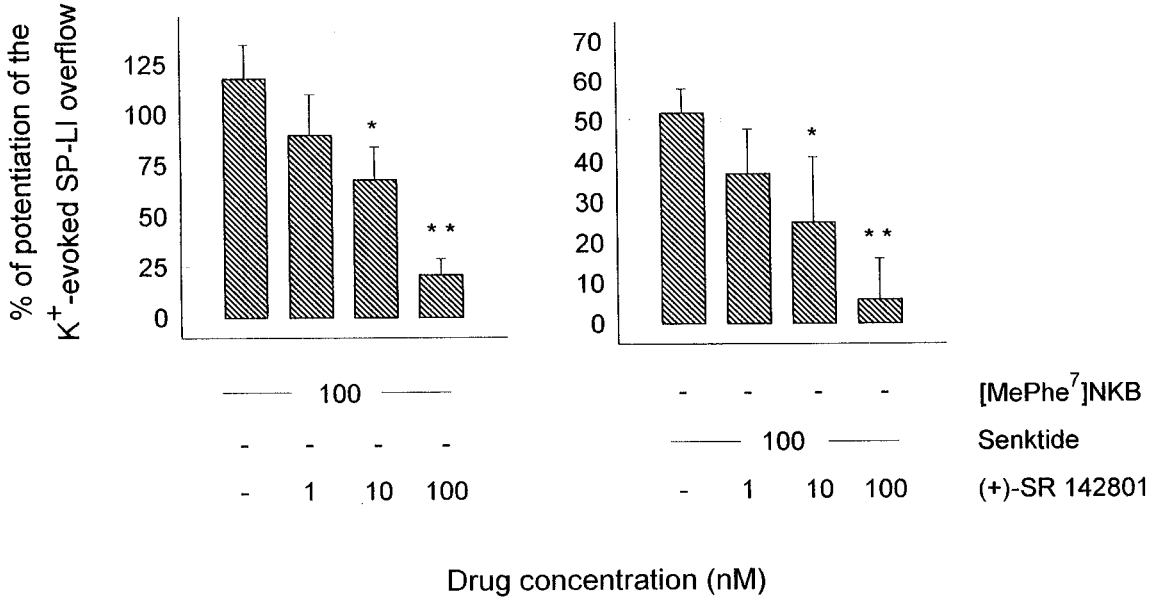

Figure 3 Antagonism by (+)-SR 142801 of the potentiation of SP-LI overflow induced by [MePhe^7]NKB (left) or senktide (right) in rat spinal cord synaptosomes. Agonists were added to the superfusion medium concomitantly with the depolarizing stimulus; (+)-SR 142801 8 min before. See Methods for calculations and other technical details. Data are means ± s.e.mean of four to six separate experiments run in triplicate. *$P<0.05$; **$P<0.001$ (two-tailed Student's *t*-test).

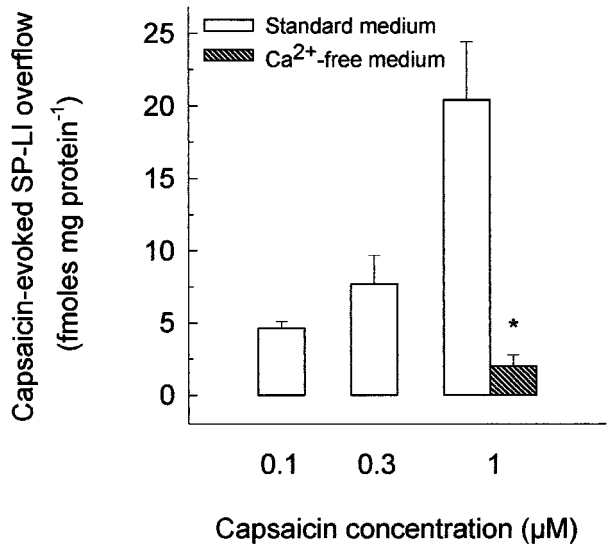

Figure 4 SP-LI overflow (fmoles mg protein^{-1}) evoked by varying capsaicin concentrations from rat spinal cord synaptosomes and its Ca^{2+} dependence. Synaptosomes were exposed for 90 s to capsaicin, after 39 min of superfusion. When appropriate, Ca^{2+}-free medium was introduced 19 min before capsaicin. See Methods for other technical details. Data are means±s.e.mean of three to six separate experiments run in triplicate. *$P<0.001$ (two tailed Student's *t*-test).

Table 2 Effect of senktide on the capsaicin-evoked SP-LI overflow from rat spinal cord synaptosomes

Drugs	*SP-LI evoked overflow (fmoles mg protein^{-1})*	n
Capsaicin (0.1 μM)	4.7±0.6	3
Capsaicin (0.1 μM) + senktide (100 nM)	5.1±0.6	3
Capsaicin (0.3 μM)	7.7±0.9	3
Capsaicin (0.3 μM) + senktide (100 nM)	7.9±1.0	3
Capsaicin (0.3 μM) + senktide (1000 nM)	8.2±0.9	3
Capsaicin (1 μM)	20±5.0	4
Capsaicin (1 μM) + senktide (100 nM)	20±3.1	4

See Methods for calculations and technical details. Values represent means±s.e.mean of data obtained in *n* separate experiments run in triplicate.

Discussion

Tachykinins act at three distinct types of receptor termed NK-1, NK-2 and NK-3 preferentially activated by SP, NKA and NKB, respectively (for reviews see Maggi *et al*., 1993; Watling & Krause, 1993; Regoli *et al*., 1994). Activation of tachykinin receptors leads to elevation of intracellular [Ca^{2+}] through the production of inositol triphosphate (see Guard & Watson, 1991 for a review).

The Ca^{2+}-dependent release of SP evoked by K^+-depolarization of spinal cord synaptosomes was potentiated by nanomolar concentrations of [MePhe^7]NKB or senktide, two selective NK-3 receptor agonists (Torrens *et al*., 1991; Maggi *et al*., 1993; Regoli *et al*., 1994; Suman-Chauhan *et al*., 1994). The effects of both agonists were prevented by the selective NK-3 receptor antagonist (+)-SR 142801 (Emonds-Alt *et al*., 1993, 1995; Beaujouan *et al*., 1997), but neither by

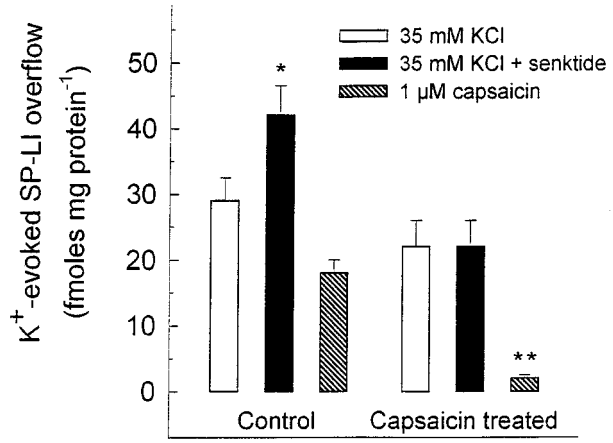

Figure 5 Effects of capsaicin pretreatment on the SP-LI overflow elicited by high-K^+ or by capsaicin and on the potentiation of the K^+-evoked overflow produced by senktide. After 39 min of superfusion, synaptosomes were exposed for 90 s to 35 mM KCl or to 1 μM capsaicin, after 39 min of superfusion; senktide (100 nM) was added to the superfusion medium concomitantly with KCl. Synaptosomes had been superfused for 15 min with 10 μM capsaicin starting at $t=10$ min of superfusion. See Methods for other technical details. Data are means±s.e.mean of three experiments run in triplicate. *$P<0.05$; **$P<0.001$ (two tailed Student's *t*-test).

RP 67580 nor MEN 11420, selective NK-1 and NK-2 receptor antagonists, respectively (Garret *et al*., 1991; Catalioto *et al*., 1998), confirming the involvement of NK-3 tachykinin receptors.

The use of synaptosomes to monitor SP release and the Ca^{2+}-dependency of the depolarization-evoked SP overflow suggest that the neurokinin released originates from axon terminals. Moreover, the characteristics of the technique employed (thin layer of synaptosomes immobilized on microporous filters and superfused in conditions known to minimize indirect effects) make it likely that the NK-3 receptors here characterized are situated on axon terminals that release SP. Dorsal horn NK-3 binding sites had been found to increase after dorsal rhizotomy, suggesting a post-synaptic localization (Yashpal *et al*., 1991). However, a recent immunoelectron microscopic study of NK-3 receptors in the rat spinal cord shows labelling not only of dendritic spines but also, although less frequently, of axon terminals of lamina II (Zerari *et al*., 1997). Our data confirm that presynaptic NK-3 receptors exist in the spinal cord, show that they are sited on SP-releasing terminals and attribute to these NK-3 receptors a possible function, that of mediating enhancement of the depolarization-evoked release of SP.

[MePhe^7]NKB displayed significantly higher efficacy and apparent affinity than senktide. These results appear in line with binding data obtained with [^{125}I]iodohistidyl [MePhe^7]NKB showing that the B_{max} values for this ligand were higher than those for [^{3}H]senktide and that [MePhe^7]NKB bound with higher affinity than senktide (Suman-Chauhan *et al*., 1994).

The tachykinin peptides have been shown to modulate the release of CNS transmitters including acetylcholine (Guard & Watson, 1991) and dopamine (Trembley *et al*., 1992). The finding that activation of NK-3 receptors sited on SP-containing terminals elicited release of SP indicates that tachykinins may directly interact with each other; in particular, endogenous NKB may enhance SP-mediated transmission. By combining electrophysiological recording with immunocytochemical detection Hu *et al*. (1997) identified SP-activated currents in rat dorsal root ganglion neurones immunoreactive

for SP, suggesting the presence of SP autoreceptors. A very recent report (Brechenmacher *et al*., 1998) shows expression of functional NK-1, NK-2 and NK-3 receptors by rat dorsal root ganglia sensory neurones in culture.

SP release evoked by high K^+ in spinal cord synaptosomes could derive from terminals of intrinsic dorsal horn neurones as well as primary afferents sensory neurones. Capsaicin, the pungent ingredient of *Capsicum* red peppers, selectively affects thin nociceptive primary afferent sensory neurones by opening cation-selective ion channels with consequent rise of intracellular Ca^{2+} (Bevan & Szolcsanyi, 1990; Holzer, 1991; Maggi, 1991; Caterina *et al*., 1997). Capsaicin was shown to evoke Ca^{2+}-dependent SP release from the central endings of sensory neurones, but not from intrinsic neurones in spinal cord slices (Gamse *et al*., 1979; Holzer, 1991). We here found that capsaicin elicited Ca^{2+}-dependent release of SP from spinal cord synaptosomes, indicating that the preparation used contains afferent neuronal axon terminals. Since capsaicin may depolarize the membrane of sensory neurones, due in part to Na^+ influx (Marsh *et al*., 1987), one may expect NK-3 receptor agonists to potentiate the release of SP induced by capsaicin as well as that induced by high-K^+. Senktide failed however to potentiate the overflow of SP produced by capsaicin. Similarly, neurotrophin-3 was found to inhibit the electrically-evoked release of SP from a rat spinal cord preparation *in vitro*, but to be ineffective on the release induced by capsaicin (Malcangio *et al*., 1997). One possible explanation for these differential effects comes from the recent observation that presynaptic receptors in general seem to modulate transmitter exocytosis strictly linked to activation of voltage-sensitive Ca^{2+} channels, but not vesicular release triggered by Ca^{2+} ions originating from other sources (Fassio *et al*., 1996). Accordingly, the reported lack of involvement of voltage-sensitive Ca^{2+} channels in the action of capsaicin (see Maggi *et al*., 1989; Maggi, 1991), in contrast with their critical role in the release evoked by electrical stimulation or K^+-depolarization, could explain the inability of neurotrophin-3 and senktide to modulate the release of SP produced by capsaicin.

On the other hand, when synaptosomes were exposed to senktide following 15 min pretreatment with capsaicin, the NK-3 receptor agonist failed to enhance the overflow of SP produced by high-K^+. Moreover, the ability of the toxin to release SP from spinal cord synaptosomes was lost after capsaicin pretreatment, indicating that the SP present in the nerve terminals of primary afferent sensory neurones had been depleted by capsaicin or the capsaicin receptor/ion channel complex had desensitized (Dray *et al*., 1989a,b; Holzer, 1991, for review). Altogether these data are compatible with the view that NK-3 presynaptic receptors are localized on central terminals of afferent neurones and not on SP-containing terminals of intrinsic dorsal horn neurones.

Little is known about the role that NKB and NK-3 receptors play in nociception. Intrathecal injections of NKB were reported to elicit antinociceptive effects mediated by NK-3 receptors (Couture *et al*., 1993). Our results showing that activation of NK-3 receptors facilitates SP release from primary afferent terminals may appear incompatible with an antinociceptive activity of NKB. On the other hand, the *in vivo* effect of NKB occurred indirectly, through release of endogenous opiates, whereas the effect here observed is direct on SP-releasing sensory neuronal terminals. The releasing effect of NK-3 receptor activation is in keeping with recent data showing that NK-3 receptor agonists caused elevation of intracellular $[Ca^{2+}]$ in cultured, capsaicin-sensitive, dorsal root ganglia sensory neurones (Brechenmacher *et al*., 1998). This is not surprising, on the other hand, since the main second messenger system coupled to activation of the three known tachykinin receptors is stimulation of phosphoinositide breakdown and elevation of intracellular Ca^{2+} (see Guard & Watson, 1991, for review). NK-3 receptors may therefore mediate different effects in the spinal cord depending on whether they are activated by endogenously released NKB or by exogenously administered agonists.

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