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SUBSTANCE P AND NEUROTENSIN: DISCOVERY, ISOLATION, CHEMICAL CHARACTERIZATION AND PHYSIOLOGICAL STUDIES

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SUMMARY

During the course of work directed towards the purification of a corticotropin-releasing factor, two other biological active substances were discovered in bovine hypothalamic extracts: one a sialogogic peptide, the other a vaso-active substance easily separated from the sialogogic agent by ion-exchange chromotography. The sialogogic peptide was subsequently characterized as substance P (SP); the other substance, also a peptide, was named neurotensin (NT). These peptides were isolated, their amino acid sequences determined (substance P; arg-pro-lys-pro-gln-gln-phe-phe-gly-leu-met-NH_2; neurotensin: < glu-leu-tyr-glu-asn-lys-pro-arg-arg-pro-tyr-ile-leu-OH), synthetic material prepared, and radioimmunoassays developed. Subsequent studies from many laboratories have yielded much information relevant to the physiological roles of these peptides. In addition to its uneven distribution throughout the CNS, SP is also present in the peripheral nervous system. Data is presented on its distribution in sympathetic ganglia and its depletion by pretreatment with capsaicin. Release of immunoreactive substance P (I-SP) has been demonstrated *in vivo* from mammalian spinal cord after activation of nociceptive afferents. Neurotensin is also unevenly distributed throughout the CNS and is present in the GI tract. Neurotensin containing cells have been demonstrated scattered throughout the small intestinal epithelium of every mammalian and avian species. The concentration of immunoreactive NT has been measured to increase in hepatic portal vein plasma after infusing a micellar solution of lipid through the small intestine of rats. Neurotensin has been demonstrated to be present in a cell line derived from a rat medullary thyroid carcinoma permitting studies on the regulation of neurotensin release from these cells in culture.

Substance P: discovery, isolation, and chemical characterization

In 1976 the discovery of a sialogogic peptide in bovine hypothalamic extracts was reported (Leeman & Hammerschlag, 1967). This peptide was subsequently isolated (Chang & Leeman, 1970) and characterized by multiple chemical and biological criteria to be the peptide designated as substance P, first described in extracts of equine brain and intestine by von Euler & Gaddum, 1931. This rather non-commital term entered the literature in 1934 (Gaddum & Schild, 1934) and gained wide acceptance. The amino acid sequence of substance P was established in 1971 (Chang, Leeman & Niall, 1971) to be arg-pro-lys-pro-gln-gln-phe-phe-gly-leu-met-NH_2 and shortly

Table 1. *I-SP content of sympathetic ganglia and the depletion by capsaicin pretreatment*

	Superior cervical	Middle and inferior cervical	Thoracic	Coeliac–superior mesenteric
Rat	70 ± 8 (4)	84 ± 14	269 ± 39 (8)	508 ± 22 (8)
Guinea pig				
Controls	258 ± 28 (8)	270 ± 26 (4)	818 ± 73 (6)	1576 ± 157 (12)
Capsaicin	29 ± 4 (5)	99 ± 12 (7)	107 ± 13 (7)	160 ± 11 (4)
Decrease (%)	89	63	87	90

I-SP values are fmol/mg protein ($\bar{x}$ ± S.E.M. of n animals). Guinea pigs were pretreated with 125 mg/kg capsaicin 8–11 days prior.

$P < 0.001$ for all ganglia.

thereafter synthetic material was prepared according to this sequence (Tregear *et al.* 1971) and found to be biologically and chemically indistinguishable from native material. The availability of synthetic material greatly facilitated biochemical, histochemical, physiological and pharmacological studies of this interesting peptide. A recent review summarizes evidence for substance P as a transmitter candidate, particularly in relation to nociceptive primary afferent fibres (Nicoll, Schenker & Leeman, 1980).

Substance P in sympathetic ganglia, distribution and sensitivity to capsaicin

In addition to its uneven distribution throughout the central nervous system, substance P has been shown to be present in various parts of the peripheral nervous system (Hökfelt *et al.* 1975; Lundberg *et al.* 1978), as well as in intrinsic neurones of the intestine (Costa *et al.* 1980), and sympathetic ganglia (Hökfelt *et al.* 1977). Gamse *et al.* (1980) studied the distribution of immunoreactive substance P (I-SP) in several sympathetic ganglia of rats and guinea pigs (Table 1) and found that pretreatment of the guinea pigs with capsaicin (a derivative of homovanillic acid) caused a marked depletion of I-SP in all ganglia examined. Male rats (Sprague Dawley, 200–250 g) and guinea pigs (Hartley, 250–350 g) were used for all experiments. Animals were killed by decapitation. The superior and the middle plus inferior cervical ganglia, the thoracic ganglia at spinal level T3-T5, and the coeliac–superior mesenteric ganglion complex referred to as coeliac ganglion were dissected under a stereomicroscope, desheathed and immediately extracted in 0·2–2·0 ml of 2 N acetic acid (4 °C). All other tissue samples were first frozen on dry ice, weighed and then extracted. SP was measured by radioimmunoassay (Mroz & Leeman, 1979). The lower limit of this assay, e.g. 12% displacement, is 1·5 fmol per assay tube. All sample dilutions were parallel to the standard curve of synthetic SP (Beckman). Many previous studies by other investigators have indicated that the action of capsaicin in the peripheral nervous system is restricted to sensory neurones. These results thus support the notion that SP fibres in sympathetic ganglia are primary sensory neurones.

In a further series of studies (Gamse *et al.* 1980) the question of whether acutely administered capsaicin would stimulate the release of I-SP from ganglionic tissue was investigated. For these studies, guinea-pig coeliac ganglia were dissected and bathed in a few drops of Krebs bicarbonate buffer. A crystal of collagenase (401 u./mg, Worthington) was placed on the ganglia, covered with another few drops of buffer and left for 15 min at room temperature. After thorough washing with fresh bu[illegible]

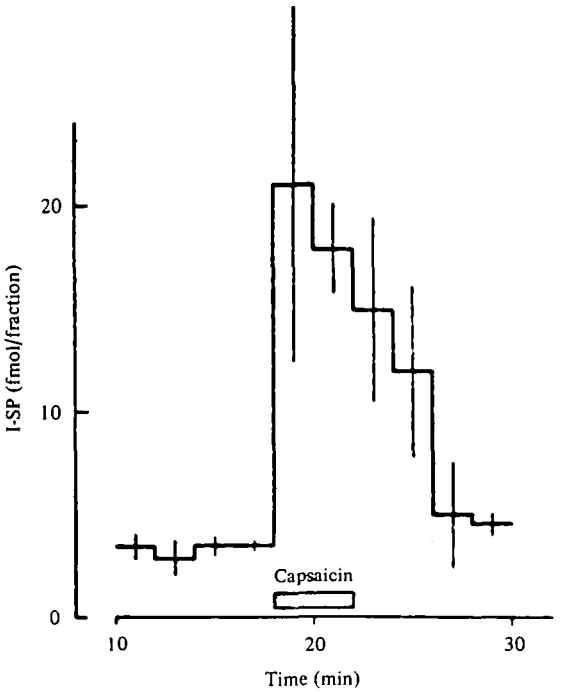

Fig. 1. Release of I-SP from individual superfused guinea-pig coeliac ganglia. Capsaicin (3.3×10^{-5} M) was added to the superfusion buffer for 4 min. $\bar{x} \pm$ S.E.M. three experiments.

the ganglia were teased apart with fine forceps and transferred into a 300 μl perfusion chamber. They were superfused with Krebs bicarbonate buffer containing 0·1% bovine serum albumin and 20 μM bacitracin at 37 °C as described previously (13). After an initial wash period of 10 min, 2 min fractions (flow rate 250 μl min^{-1}) were collected into an equal volume of 4 N acetic acid and lyophilized.

A low resting release of I-SP from the coeliac ganglion was found at the limit of the assay. Superfusion with buffer containing $3{\cdot}3 \times 10^{-5}$ M capsaicin resulted in an immediate 6·4-fold increase in I-SP release (Fig. 1). The total evoked release constituted $1{\cdot}3 \pm 0{\cdot}3\%$ ($n = 3$) of the I-SP content of the ganglia. Superfusion with a calcium-free buffer containing 2 mM-Co^{2+} completely inhibited the capsaicin-evoked release ($n = 2$). While it is possible that the SP-positive fibres in sympathetic ganglia morphologically resembling nerve endings are sensory detectors, it is more likely that they are presynaptic to principal ganglion cells. These endings may be collaterals of primary sensory SP neurones. If this idea is correct, stimulation of the sensory endings of these fibres – by modalities and in tissues yet to be determined – would then cause release of SP not only in the CNS but also in sympathetic ganglia thereby affecting the activity of postganglionic neurones.

Release of substance P from mammalian spinal cord in vivo

Although considerable information supports a role for substance P in the transmission of painful stimuli (see review Nicoll, Schenker & Leeman, 1980), evidence that substance P is actually released *in vivo* after activation of chemo-sensitive sensory

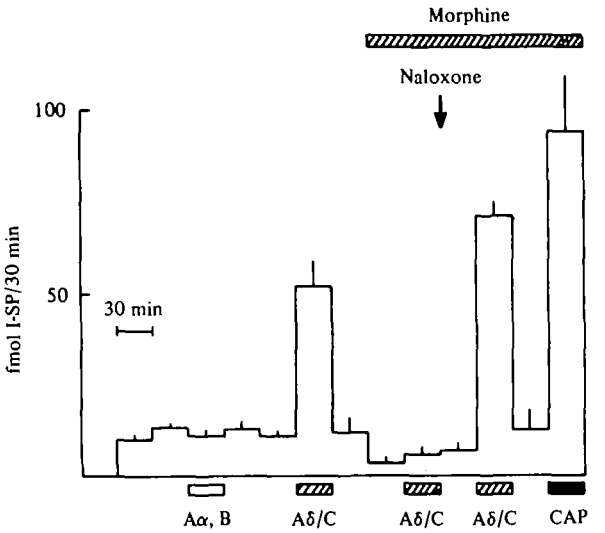

Fig. 2. Release of I-SP from superfused cat spinal cord in response to sciatic nerve stimulation and capsaicin (CAP). Superfusate in all experiments consisted of NaCl, 151·1 mM; KCl, 2·6 mM; Mg_2SO_4, 0·9 mM; $CaCl_2$, 1·3 mM; $NaHCO_3$, 21·0 mM; K_2HPO_4, 2·5 mM; and gassed with 95 % O_2 and 5 % CO_2 before perfusion. Three ml perfusion samples were collected in glacial acetic and (final concentration of 2 N) immediately frozen and lyophilized. Samples were reconstituted in 1·0 ml phosphate buffered saline (pH 7·4) containing 0·1 % gelatin and aliquots of each fraction were used to determine the content of substance P by radioimmunoassay using antibody R6P with a sensitivity of 2 fmol/sample. Serial dilutions of synthetic substance P and immunoreactivity in superfusate samples produced parallel dilution curves. Each value is the mean ± S.E.M. from four separate experiments.

neurones or by electrical activation of small-diameter primary sensory afferents was lacking until some recent experiments of Yaksh *et al.* (1980).

Cats were anaesthetized with chloralose-urethane (100 mg/kg) and prepared with a tracheal tube and jugular and carotid catheters. An incision was made in the cisterna magna and the perfusion cannula was inserted after retraction of the subarachnoid layer. The perfusion cannula consisted of a length of polyethylene PE-90 (1·0 mm OD) tubing through which was passed a length of polyethylene PE-10 tubing. The PE-10 served as inflow cannula while the PE-90 was used as collection cannula. Both cannulae were inserted through the retracted incision in the cisternal membrane 37–40 cm down the spinal cord (upper sacral region). The PE-90 cannula was then retracted 10 cm, leaving the tip of the PE-10 cannula 27–40 cm deep. Perfusion was therefore localized to the upper sacral and lumbar spinal cord. The position of the inflow and outflow cannulas was determined by X-rays after infusion of radio-opaque dye.

The sciatic nerve was exposed and prepared for stimulation and recording of the compound action potential. Stimulation of the nerve performed with rectangular impulses (3–4 V, 0·5 ms for Aα and Aβ fibres and 40–50 V, 0·5 ms for recruitment of A§ and C fibres).

It was possible to detect a significant release of substance P from cat spinal cord in the absence of any evoking stimulus. Between 7 and 15 fmol of substance P were detected in each 30 min collection period with very little variation during the course of the experiment (Fig. 2). To discover whether substance P is released from the spinal cord following activation of specific afferent fibre populations, the cat sci

nerve was stimulated bilaterally, while superfusing the lumbar spinal cord receiving sensory input from the sciatic nerve. During stimulation, the compound action potential was monitored to determine the stimulus intensity required to activate A$\alpha\beta$ fibres alone, and that required to recruit Aδ and C fibres. At stimulus intensities sufficient to recruit only low threshold A$\alpha\beta$ afferents, there was no significant increase in the release of substance P (Fig. 2). The collaterals of large myelinated fibres that are activated by low-intensity stimuli terminate within deeper laminae of the dorsal horn. By superfusing the surface of the spinal cord, therefore, we cannot exclude that substance P is released from the terminals of low-threshold afferents but cannot diffuse into the spinal superfusate. Increasing the stimulus intensity to activate Aδ and C fibres produced a $4{\cdot}9 \pm 0{\cdot}6$-fold (mean $\pm$ S.E.M.; $n = 4$) increase in substance P release. In the fraction following stimulation, the release of substance P returned to pre-stimulation values.

Following superfusion with 5×10^{-4} M morphine (equivalent to 520 μg morphine superfused over a 30 min period) bilateral stimulation of the sciatic nerve at intensities that clearly caused the release of substance P before the addition of morphine now failed to increase the release of substance P. Following intraperitoneal injection of naloxone (1 mg/kg) stimulation of the sciatic nerve at the same intensity, in the continued present of intrathecal morphine, fully restored the evoked release of substance P. Superfusion of the cat spinal cord with 5×10^{-4} M capsaicin produced a greater than 10-fold increase in the release of substance P ($n = 5$ experiments). Since the actions of capsaicin in the spinal cord seem to be restricted to nociceptive primary sensory neurones, the demonstration of a capsaicin-evoked release of substance P from cat spinal cord, *in vivo*, provides further evidence that substance P may be involved in the transmission of noxious peripheral stimuli. The precise site(s) of action of morphine in inhibiting the release of substance P in these experiments is, of course, unclear.

Neurotensin: discovery, isolation from bovine hypothalamic tissue and chemical characterization

During the course of purification of substance P from bovine hypothalamic extracts, another vaso-active substance was detected in the eluate of an ion-exchange column that was clearly separable from the sialogogic activity (Fig. 3). It was found that this vasodilatation was associated with a transient hypotension and that this activity was susceptible to proteolytic digestion. Using this biological property to monitor purification procedures, the causal peptide was isolated (Carraway & Leeman, 1973) and named neurotensin because of its presence in neural tissue and its ability to affect blood pressure. The amino acid sequence of this peptide was determined to be < glu-leu-tyr-glu-asn-lys-pro-arg-arg-pro-tyr-ile-leu-OH by a combination of Edman degradation and carboxypeptidase treatment of papain-generated fragments of the molecule (Carraway & Leeman, 1975*a*). Synthetic material was prepared (Carraway & Leeman, 1975*b*) and used to generate radioimmunoassays (Carraway & Leeman, 1976*a*).

Isolation of neurotensin from gastrointestinal tissue

With the use of these radioimmunoassays the distribution of neurotensin was determined in acid-acetone extracts of various rat tissues and found to be present in

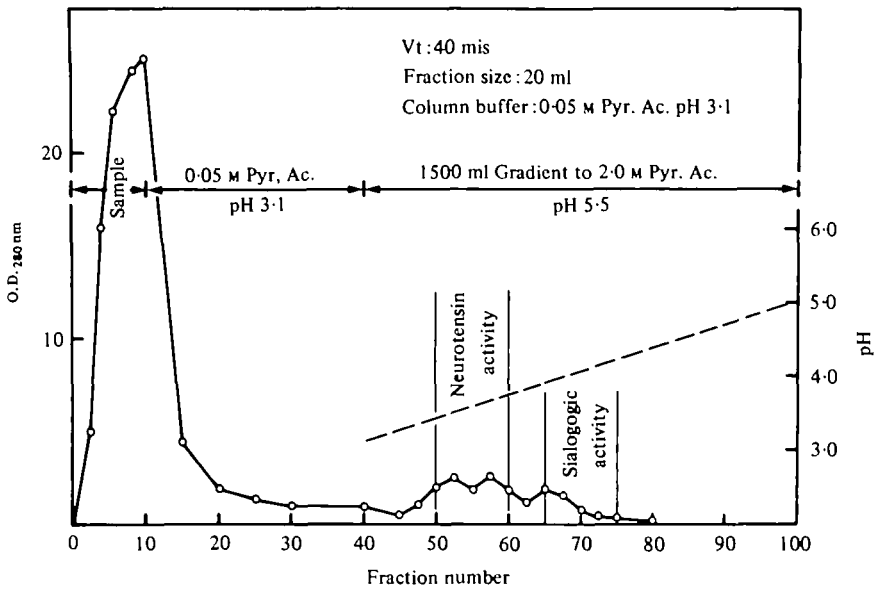

Fig. 3. Ion-exchange chromatography of a bovine hypothalamic extract on sulfoethyl Sephadex. Neurotensin and substance P (sialogogic activity) were detected using bioassay; protein concentration was monitored at 280 nm. Pyr Ac, pyridine acetate.

approximately the same concentration in the full thickness jejuno-ileum (50–60 pmol g^{-1} wet weight) as in the hypothalamus (Carraway & Leeman, 1976*b*). Subsequent purification of immunoreactive neurotensin from bovine small intestine (Kitabgi, Carraway & Leeman, 1976) yielded a peptide indistinguishable from that of hypothalamic neurotensin.

In collaboration with the late Dr Robert Williams, we studied the distribution of neurotensin in fresh post-mortem human mucosal scrapings and found that, in humans, intestinal neurotensin increases in the distal jejunum and ileum, in agreement with findings in other species. Sufficient material was collected from scrapings of the distal mucosa to permit us to isolate human intestinal neurotensin (Hammer *et al.* 1980) and to determine its amino acid composition. The peptide was found to be chemically and biologically indistinguishable from bovine neurotensin. The pure material ran as a single peak on reverse-phase high-pressure liquid chromatography and was identical in elution volume with synthetic NT. Only a part of the amino acid sequence of human NT could be determined because of the limited amount of pure peptide. However, digestion of human and bovine NT with papain, followed by separation of the peptide fragments on reverse-phase high-pressure liquid chromatography and determination of the amino acid composition of the separated fragments, yielded identical results for the two molecules, suggesting that the sequence is also identical. Demonstration of the identity of human neurotensin and the bovine peptide to which all antisera thus far reported have been raised means that a reliable radioimmunoassay for bovine neurotensin will give accurate measurements of the human peptide as well.

Neurotensin cells in the intestine

Several groups have demonstrated neurotensin-containing cells scattered throughout the small intestinal epithelium of many mammalian and avian species (Helmstaedter *et al.* 1977; Orci *et al.* 1976; Polak *et al.* 1977; Sundler *et al.* 1977). In all species investigated, these cells appear to be of the open variety, with apices in contact with the lumen and with the neurotensin granules concentrated toward the base of the cell. The orientation of these neurotensin cells raises the interesting possibilities that they are true endocrine cells and that neurotensin is released into the circulation in response to a stimulus communicated the cell by way of the lumen. In support of this notion, recent findings in our laboratory (C. Ferris, R. Hammer, R. Carraway & S. E. Leeman, unpublished observations) have demonstrated that the infusion of a micellar solution (2·4 mM-Na-taurodeoxycholate, 0·6 μM oleic acid 0·3 μM monolein in 0·9% saline) *in vivo* through the small intestine of rats caused a significant increase in the concentration of immunoreactive NT in hepatic portal vein plasma, whereas these levels were unchanged following infusion of 0·9% or 2·7% saline, 8·5% aminosol (amino acid mixture) or 5·6% dextrose.

Neurotensin production by a line of rat medullary carcinoma cells

A rMTC 6–23 cell line derived from a calcitonin producing rat medullary thyroid carcinoma was discovered to also synthesize and secrete neurotensin (Zeytinoglu *et al.* 1980). The immunoreactive neurotensin extracted from these cells was isolated and found to be chemically and biologically indistinguishable from synthetic neurotensin. Both high K^+ and high Ca^{2+} stimulated the release of neurotensin into the medium. This cell line may provide a very useful system in which to study the biosynthesis and regulation of release of neurotensin.

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