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Substance P and related peptides in porcine cortex: whole tissue and nuclear localization

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Abstract

A large portion of the knowledge that has been gathered on the distribution of neuropeptides in neural tissues is based on findings obtained with immunocytochemistry and radioimmunoassay. However, these methods give limited structural information about the peptides being studied. Using porcine cortex as a model tissue, we combined immunoaffinity chromatography with reversed-phase high-performance liquid chromatography, radioimmunoassay, and matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF-MS). We determined the molecular nature of the peptides contributing to the substance P-like immunoreactivity measured in extracts of whole tissue and cell nuclei. In addition to substance P(1–11), other peptides were extracted using this protocol. The presence of SP(1–11) was confirmed through post-source decay analysis. These results illustrate the usefulness of MALDI-TOF-MS in the characterization of neuropeptides from biological tissues. © 1998 Elsevier Science B.V.

Keywords: Immunoaffinity chromatography; Substance P; Peptides

1. Introduction

Substance P is an undecapeptide amide (RPKPQQFFGLM–NH_2) [1] that is formed by post-translational processing of the preprotachykinin (PPT) precursors α-, β- and γ-PPT [2]. Substance P has previously been identified in neural tissues [3–13] and pituitary cell nuclei [14] through immunocytochemistry and radioimmunoassay (RIA). Although sensitive, these methods give limited structural information about the molecular species studied.

In this study, we combined immunoaffinity chromatography with reversed-phase high-performance liquid chromatography (RP-HPLC), RIA, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) in order to determine the molecular nature of the peptides contributing to the substance P-like immunoreactivity (SP-LI) found in porcine cerebral cortex. Because the yield of different SP-like immunoreactive peptides has been shown to depend on the pH of the extraction medium [5,6], we used an acidic and a neutral extraction. In order to enable detection of variant SP-like immunoreactive peptides, two different immunoaffinity columns were prepared, one with an N-terminally directed antiserum, the other with a C-terminally directed antiserum.

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2. Experimental

2.1. Tissue extraction

Porcine brains were obtained immediately after death at the local abattoir, and transported on ice to our laboratory. Cortex was dissected out and frozen immediately at −70°C. For the extraction of whole tissue, the sample was weighed and boiled for 15 min in 1:1 (m/m) of either 0.5 *M* acetic acid (acidic extraction) or 0.05 *M* sodium phosphate buffer pH 7.4 (neutral extraction) and was homogenized using a Kinematica (Luzern, Switzerland) homogenizer. The use of phosphate buffer instead of pure water for extraction of peptides from biological samples has been shown to give superior yields of neuropeptides in our laboratory. Approximately 15 g of cortex was extracted in each procedure. After centrifugation at 3000 *g* and 4°C for 10 min, the supernatant was removed and dried by vacuum centrifugation (Speedvac, Savant). The isolation of porcine cortical nuclei was performed as described by Buu [15]. In short, the cortex was weighed and homogenized using ten up and down strokes in a 40 ml glass homogenizer. The homogenization buffer contained 0.32 *M* sucrose, 10 m*M* Tris, 10 m*M* $MgCl_2$ and 0.1% Triton X-100, pH 7.4 and was kept at 4°C. After centrifugation, the pellet was rehomogenized in the buffer and mixed 1:1 (v/v) with 2.2 *M* sucrose. The mixture was layered over 2.2 *M* sucrose and centrifuged at 75 000 *g* for 60 min at 4°C. The resulting nuclear pellet was examined by light microscopy. The pellets were frozen at −70°C until analysis. The pellets were extracted in 1.0 ml of either 0.5 *M* acetic acid or 0.05 *M* sodium phosphate buffer pH 7.4 for 4 h at 4°C. The mixture was centrifuged and the supernatant dried as above.

2.2. Immunoaffinity chromatography

Two separate immunoaffinity chromatography columns were prepared. In short, total immunoglobulin G (IgG) antibodies were each purified from the unprocessed antisera SPN1 [6] and SP10 [5] by chromatography on a column packed with Protein G Sepharose gel (Pharmacia, Uppsala, Sweden). A substance P-Sepharose column (SP-column) was prepared by coupling 50 mg synthetic substance P (Ferring, Malmö, Sweden) dissolved in 100 ml of 0.1 *M* $NaHCO_3$, pH 6.0 to 1.4 g of Activated CH Sepharose 4B (Pharmacia) for 45 h at 20°C. The coupling reaction was monitored by HPLC and RIA. Approximately 50% of the peptide was coupled. The IgG preparations (SPN1 and SP10) were chromatographed on the SP-column in separate runs. About 20% of the total IgG from the both antisera was bound, as determined according to the BCA protein method (Pierce, Rockford, IL, USA). The specific SP-antibodies were each coupled to CNBr-activated Sepharose 4B (Pharmacia) following the manufacturer's instructions. In this step, more than 90% of the antibodies were coupled. The dried tissue extracts were dissolved in 6.0 ml of a buffer containing 0.05 *M* sodium phosphate and 0.5 *M* NaCl, pH 7.4 and loaded on the immunoaffinity column (90×5 mm). The column was washed once with the same buffer and once with buffer without NaCl. The samples were eluted with 0.2 *M* acetic acid, and fractions obtained were dried by vacuum centrifugation (Speedvac, Savant).

2.3. RP-HPLC

The immunoaffinity-purified peptides were reconstituted in 125 μl mobile phase A. Solution A was 0.1% trifluoroacetic acid (TFA) in water and solution B was 0.1% TFA in acetonitrile. Separation of peptides was achieved using the SMART System (Pharmacia) fitted with a Poros RH column, 30×2.1 mm I.D. (PerSeptive Biosystems, Cambridge, MA, USA). The column was equilibrated with 5% mobile phase B at a flow-rate of 1000 μl/min. Separation of peptides was achieved by 5% B for 30 s, followed by a linear gradient from 5 to 38% B over 5 min, and from 38% to 60% B over 30 s. All of the solvents used were of HPLC purity. The flow-rate was 1 ml/min. Fractions were collected at 15 s intervals and dried by vacuum centrifugation.

2.4. Radioimmunoassay

Competitive RIA using an antiserum against synthetic porcine SP (Cambridge Research Biochemicals, Northwich, UK), at a final dilution of 1:80 000 was employed [16]. The antiserum (SP2) reacts 100% with SP and oxidized SP, 150% with SP

(3-11), and 85% with SP (4-11), but does not cross-react with shorter fragments of SP, neurokinins or neuromedins [5,6]. The limit of detection (LOD) of SP was 5 pmol/l. Dried HPLC fractions were reconstituted in 100 μl of an assay buffer containing 0.25% human serum albumin, 500 KIU (kallikrein inactivating units)/ml Trasylol (Bayer, Leverkusen, Germany), 6.7 m*M* EDTA (Merck, Darmstadt, Germany) and 0.05 *M* sodium phosphate pH 7.4. ^{125}I-[Tyr^{8}]-SP was used as the radioligand. The radioligand (20 000 cpm) was added after 24 h incubation at 4°C. The total incubation volume was 500 μl. The final dilution of the antiserum gave 50±5% binding of radioligand. Following 48 h incubation at 4°C, free and bound tracer were separated using dextran- coated charcoal. The inter-assay variation was <15% and the intra-assay variation was <5%.

2.5. Mass spectrometry

All mass spectra were acquired using a Reflex MALDI-TOF-MS system (Bruker-Franzen Analytik, Bremen, Germany). The instrument is equipped with a nitrogen laser (337 nm), a LeCroy digitizer and a reflectron. In the reflectron mode, the flight path is 280 cm. The spectra were acquired in the reflectron mode with continuous extraction of analyte ions at an accelerating voltage of 15.6 kV. The matrix used was α-cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO, USA). An analyte-to-matrix ratio of about 1 to 50 000 was used. The post-source decay spectrum was acquired with delayed extraction of analyte ions at an initial accelerating voltage of 28 kV and a reflectron voltage of 30 kV. The width of the ion gate was set at 500 ns. The spectra are an average of 50 to 100 laser shots. The spectra were mass-assigned using the Bruker software on the Sun Sparcstation using external calibration.

3. Results

3.1. SP-LI in cortex and cortical nuclei purified by immunoaffinity chromatography with N-terminally directed SP-antiserum (SPN1)

As seen in Fig. 1A, similar HPLC–RIA profiles were obtained for whole tissue samples of cortex extracted according to the acidic and neutral protocols. Extraction in acidic medium yielded at least twice the amount of SP-like immunoreactive material as in extraction in neutral medium. The material yielded by acidic extraction of porcine cortex was sufficient to confirm the presence of substance P and oxidized SP in HPLC fraction number 12 by using MALDI-TOF-MS. Mass spectrometric analysis of SP-immunoreactive fraction number 12 revealed a mixture of peptides that included the $(M+H)^{+}$ ions at *m*/*z* 1349 and 1365 (Fig. 3), that are compatible with SP(1–11) and SP(1–11)$_{ox}$, respectively. The identity of the peptide at *m*/*z* 1349 was confirmed through post-source decay (PSD) analysis as SP(1–11) (Fig. 4; see Table 1 for other mass data). PSD refers to the unimolecular dissociation of the precursor ion that occurs in the first field-free region between the ion source and the reflectron [17].

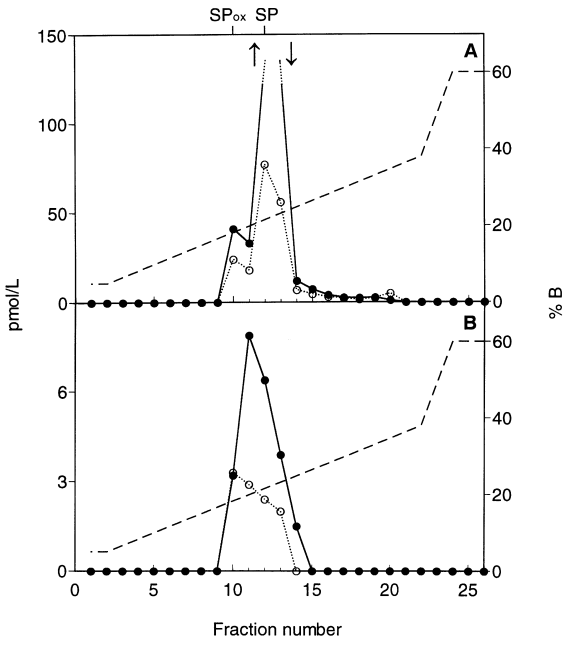

Fig. 1. HPLC–RIA profile for porcine cortex (A) and cortical nuclei (B) extracted and purified by immunoaffinity chromatography with N-terminally directed SP-antiserum (SPN1). The arrows indicate the upward continuation of the curve. Solid points indicate values obtained using acidic medium for extraction, and open points values obtained using neutral medium. Elution volumes of the peptide standards SP(1–11) and SP(1–11)$_{ox}$ are indicated at the top of the chart.

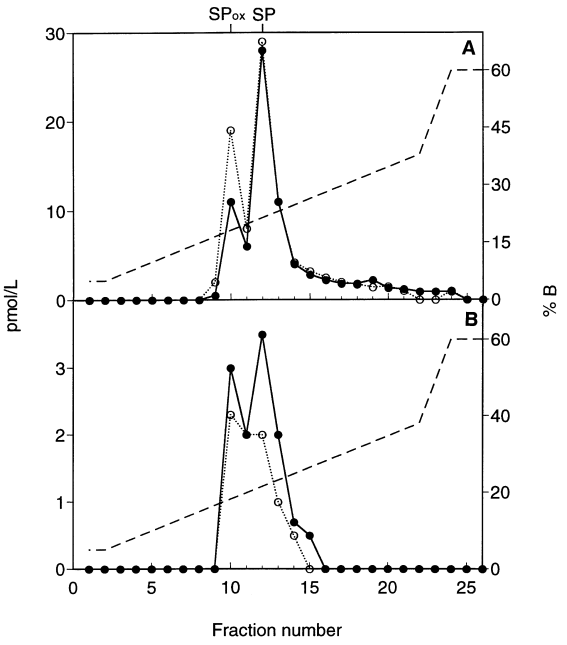

Fig. 2. HPLC–RIA profile for porcine cortex (A) and cortical nuclei (B) extracted and purified by immunoaffinity chromatography with C-terminally directed SP-antiserum (SP10). Solid points indicate values obtained using acidic medium for extraction, and open points values obtained using neutral medium. Elution volumes of the peptide standards SP(1–11) and SP(1–11)$_{ox}$ are indicated at the top of the chart.

Because the product ions have different kinetic energies than the precursor ion, they can be identified using a reflectron. The amino acid sequence of a peptide is established through the series of product ions formed, as well as by comparison of the PSD spectrum of the unknown peptide with the PSD spectrum of the synthetic peptide. The peptide fragment ion nomenclature used is based on that proposed by Roepstorff and Fohlman [18], as modified by Biemann [19]. In short, small letters symbolize dissociation sites in the precursor ion, and subscripts denote the number of amino acid residues in the ion.

The HPLC–RIA profile of SP-LI in the cortical nuclei (Fig. 1B) differed somewhat from that seen for cortex tissue. The overall yield of SP-LI per gram of tissue was as expected much lower in the nuclear fraction than in the cortex tissue extract. The acidic and neutral extraction of the nuclei yielded similar overall quantities of material. However, neutral extraction favored the yield of compounds eluting in fraction 11, whereas acidic extraction gave a higher yield of components in fraction 10. The yield of material at the elution position of SP (synthetic) itself was low, indicating that the major portion of SP-LI found in the nuclear fraction is due to peptides other than SP(1–11). The low amounts of material yielded through the extraction of the cortical nuclei precluded analysis through MS.

3.2. SP-LI in cortex and cortical nuclei purified by immunoaffinity chromatography with C-terminal directed SP-antiserum (SP10)

As seen in Fig. 2A, similar HPLC–RIA profiles were obtained for cortex samples treated according to the acidic or neutral protocols. Also, similar amounts were obtained by using either method. However, the yield was much lower using the SP10 column than the SPN1 column. Most of the SP-LI eluted at the same position as SP(1–11) (fraction 12) or SP(1–11)$_{ox}$. Characterization of the SP-LI in cortical nuclei using this antiserum gave HPLC–RIA profiles (Fig. 2B) that were similar to those obtained for whole tissue extracts, but at much lower quantities. Neither cortex tissue nor cortical nuclei yielded sufficient material for mass spectrometric analysis.

4. Discussion

We were able to confirm the presence of SP(1–11) and SP(1–11)$_{ox}$ in samples derived from whole tissue porcine cortex through affinity chromatography, RIA and MALDI-TOF-MS. MALDI-TOF-MS is a technique that is tolerant of salts and other biological contaminants, but the use of the prepurification techniques described here were found necessary in order to detect SP in the cortical extracts. Several other SP-LI immunoreactive compounds at various molecular masses are also detected in the whole tissue samples through MS, indicating that the SP-LI measured in this tissue is at least in part derived from cross-reactive peptides. The relative yields of different peptides in this tissue appear to be affected by the pH during peptide extraction; those data confirm our previous results [5]. It is interesting

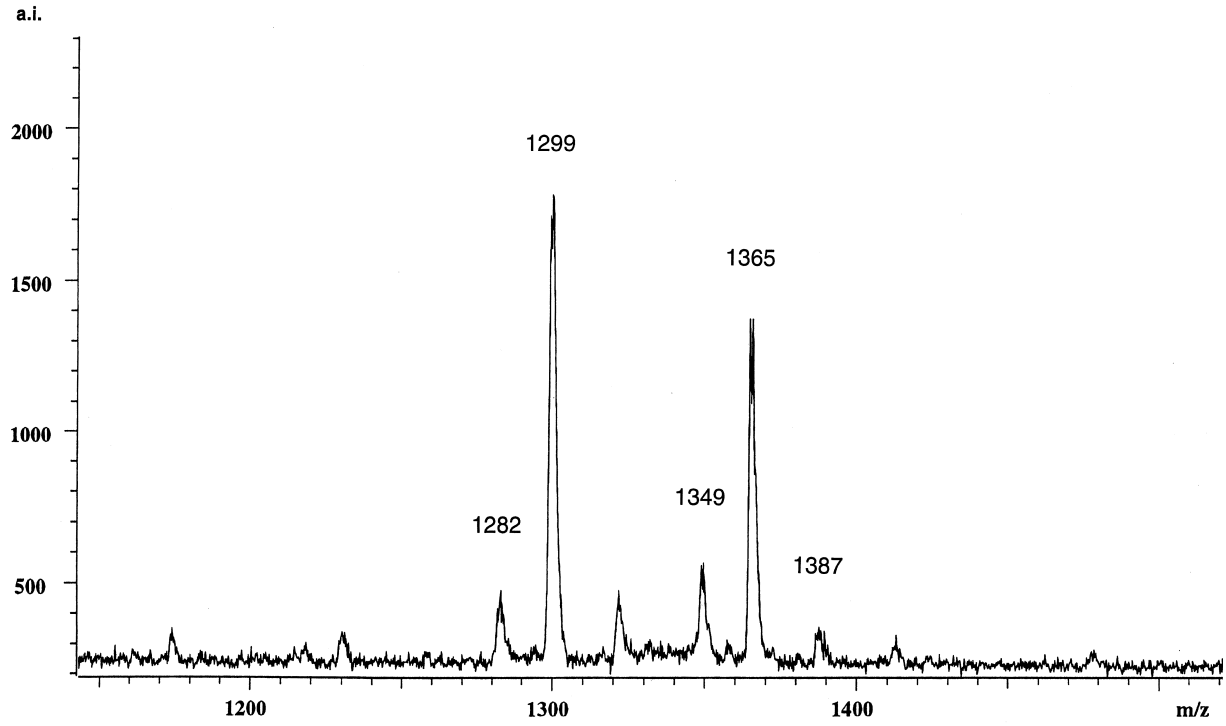

Fig. 3. The $(M+H)^+$ ions that are observed by MALDI-TOF-MS in HPLC fraction 12 from porcine cortex extracted in acidic medium and purified by immunoaffinity chromatography with the SP-antiserum SPN1. a.i.: Intensity (arbitrary units).

to note that the immunoaffinity capture yielded almost twice the amount of SP-like material using acidic extraction compared to neutral (Fig. 1A and B). However, immunoaffinity capture using the SP10 column gave similar yields for the acidic and neutral procedures (Fig. 2A and B). This difference may be explained by the presence of several peptides in these peaks and different binding characteristics and specificities between the two antisera.

The SP-LI measured in the nuclear fraction of porcine cortex showed similar HPLC–RIA profiles as in the whole tissue. SP-LI eluting earlier than either SP(1–11) and $SP(1–11)_{ox}$ was only identified in the case of cortical nuclei extracted using the neutral protocol and purified using the SPN1 column (Fig. 1B). It appears that the SP-LI in cortical nuclei, as in whole tissue extracts, consists of SP and oxidized SP; however, some other cross-reactive material may reside there as well. Although several different types of peptide-like immunoreactivity have been detected in the nuclei of different cell types [20,21], the chemical nature, structure, and possible function of nuclear neuropeptides remains elusive. In order to determine the amino acid sequences of these peptides using our methods, larger amounts of material will need to be extracted and analyzed using

Table 1
Fragment ions from the peptide at m/z 1349 observed by PSD analysis

	H-R[1]	P	K	P	Q	Q	F	F	G	L	M-NH_2
a-17	112				562	690	837	985		1155	
a		226	354		579	707	854	1002			
b-17		237	365			718					
b		254	382		607	735					

[1] Amino acid sequence.

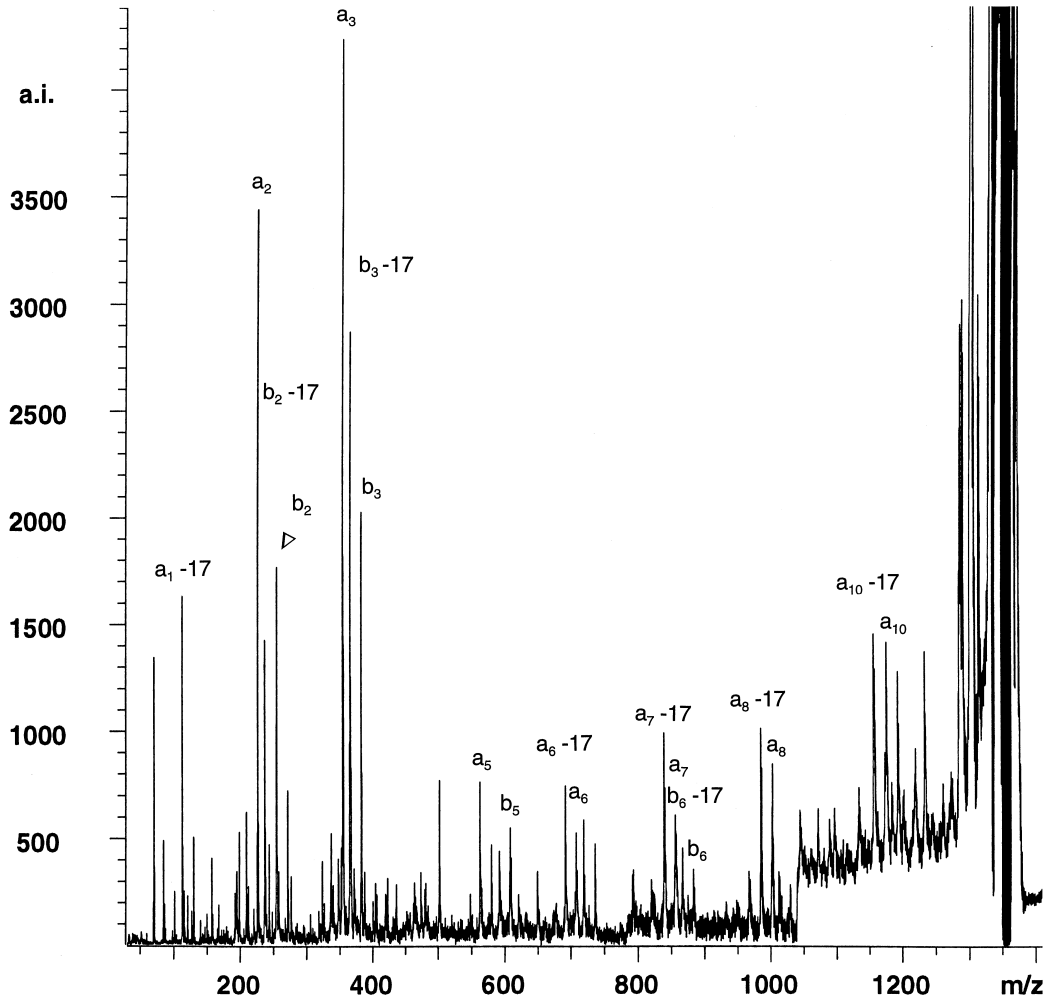

Fig. 4. PSD spectrum of the peptide found at m/z 1349. a.i.: Intensity (arbitrary units) See also the mass data tabulated in Table 1.

MS and PSD analysis. This aspect is pertinent to studies involving the quantitation of SP, because related peptides that are cross-reactive with anti-SP antisera have been discovered in biological samples taken from various species in the normal and diseased state. For instance, higher-molecular-mass species of SP, which may be N-terminally extended forms of the molecule, have been found in human cerebrospinal fluid [22], rat brain [5,23], pituitary [24], superior cervical ganglion [25] and chick dorsal root ganglia [26]; also, shorter but nonetheless immunoreactive fragments have been identified in tumors [27] and in the substantia nigra [28]. Whereas the function of the higher-molecular-mass SP is unknown, some of the shorter fragments of SP may have opioid agonist activity [29].

We were unable to detect larger-molecular-mass forms of substance P that have been reported to be found in other tissues. It may be that such forms of SP are localized in other parts of the central nervous system of this species besides the cortex, or that the antibodies used in this experiment were unable to detect them after being coupled to the immunoaffinity column. Changes in binding characteristics have been observed for monoclonal antibodies after immobilization onto solid-phase matrices [30], and this phenomenon is likely to occur with all types of antibodies.

In summary, these results illustrate the usefulness of MALDI-TOF-MS as a complement the conventional methods used in the characterization of neuropeptides from biological tissues.

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