Distribution and Characterization of VIP-Related Peptides in the Rat Spinal Cord

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Abstract—The possible existence in the rat spinal cord of a peptide related to VIP, VIP(22-28), has been evaluated. VIP contains paired basic amino acid residues at which posttranslational cleavage of these peptides might occur. The lumbo-sacral region of rat spinal cord had the most VIP(22-28)-like immunoreactivity (ir-VIP(22-28)). Chromatographic analysis of spinal extracts showed that ir-VIP(22-28) consisted of two major peaks, one eluting as authentic VIP(1-28) and the other as VIP(22-28). HPLC confirmed these results, revealing the presence of intact VIP(1-28) and two or more less hydrophobic peptides, one of which corresponded to authentic VIP(22-28). The other two components found have not yet been identified. Further studies are necessary to provide information on the biological significance of VIP(22-28).

Introduction

The spinal cord has a variety of peptide neurotransmitter systems. It contains vasoactive intestinal polypeptide (VIP), substance P, Peptide-Histidine-Isoleucine (PHI), CCK, neuropeptide, and opioid peptides (1, 2, 3, 4). Immunohistochemical analysis of the distributions of VIP and PHI, show that they are not always present in tissues in equimolar concentrations (7, 8). This suggests differential processing of their precursor which is probably tissue-specific. Furthermore, the existence of some pairs of basic amino acids (Arg14-Lys15; Lys20-Lys21) within the VIP-sequence suggests that VIP may be cleaved internally.

We have recently demonstrated the presence of a heptapeptide, VIP(22-28), produced by internal cleavage of VIP at the paired Lys residues in position 20 and 21 (9), in the rat cerebrum. VIP (22-28)-like immunoreactivity comprised about 34% of the total VIP(1-28)-immunoreactivity in the cortex, midbrain and hippocampus when an
antiserum which recognizes both VIP(1-28) and shorter VIP fragments was used to assay VIP immunoreactivity.

In the present paper we looked for VIP(22-28) in the spinal cord of the rat. We examined the longitudinal and dorsoventral distribution of VIP(22-28) and VIP(1-28) in the rat spinal cord by means of RIA, using a polyclonal antiserum raised against rat VIP(22-28) (9). The immunoreactivities detected were further studied by gel filtration and reversed phase liquid chromatography in order to characterize the molecular forms of VIP present.

Our results show VIP(22-28), together with intact VIP, is present in the spinal cord of the rat.

**Methods**

**VIP(22-28) antiserum**

Peptide, H-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH₂, VIP(22-28), was prepared by solid phase synthesis. Protected peptide was synthesized manually on a benzoylamine resin according to a standard procedure (10). Final deprotection and cleavage was performed with anhydrous HF containing 10% anisole. The purity and identity of the peptide was monitored by reversed phase HPLC and by aminoacid analysis (for complete method see ref. 9).

Antiserum against VIP(22-28) was raised by immunization of Male New Zealand white rabbits at approximately two week intervals with about 50μg of VIP(22-28) conjugate with bovine thyroglobulin in Freund's complete adjuvant.

**Radioimmunoassay procedures**

VIP(22-28) and VIP(1-28) were iodinated by the chloramine T method (11) and purified by microcolumn Sep-Pak C18 reversed phase chromatography. The labeled peptides were stable for 2-3 months at -20°C. Radioimmunoassays were carried out in 0.15M sodium phosphate buffer (pH 7.4), with incubation for 18-24h at +4°C. The reaction was terminated by the addition of 1.0ml of charcoal suspension and bound peptide separated by centrifugation and estimated essentially as described previously (9).

The VIP(22-28) antiserum, AH78, was used at a final dilution of 1:6000 in an assay volume of 300μl (IC₅₀ for VIP(22-28) was about 60fmol/assay tube). The AH78 antiserum showed full cross-reactivity with VIP(22-28), essentially complete cross-reactivity with VIP(1-28), but it did not crossreact with PHI-related peptides (9).

A second antiserum recognizing VIP(1-28) was purchased from Calbiochem (La Jolla, Ca). Using this antiserum in a typical assay, the IC₅₀ for VIP(1-28) was about 30fmol/assay tube. This antiserum recognized a central region of VIP(1-28), with 0.1% cross-reactivity for PHI-related peptides and less than 0.03% cross-reactivity for VIP(22-28) (9). A higher immunoreactivity in the VIP(22-28) RIA than in the VIP(1-28) RIA indicates the presence of VIP(22-28) and related peptides in the spinal cord extracts. Values reported were means of assays of samples from 10-15 rats and they were subjected to statistical analysis, by means of the Newman-Keuls Test.

**Tissue dissection and extraction**

Adult male Spargue-Dawley rats, weighing 220-240g were killed by decapitation. The rat spinal cords were removed by inserting a 18G needle connected to a 20ml syringe and pushing cold saline into the spinal canal from the caudal end. The spinal cord was pushed, completely intact, from the rostral end of the spinal canal.

Each issue was dissected into six spinal segments: dorsal and ventral cervical segments (SC CERV D and V), dorsal and ventral thoracic segments (SC THOR D and V), and dorsal and ventral lumbo-sacral segments (SC LUM-SAC D and V).

The tissues were rapidly weighed and homogenized in 10ml/g of 0.1M acetic acid at 95°C, and mantained at this temperature for 10min. Aliquots of the initial homogenate were set aside at -20°C for protein assay, and the extracts were cooled and centrifuged at 12500g for 20min. The supernatants were removed and stored at -20°C prior to chromatographic fractionation or assay for VIP(22-28) or VIP(1-28) immunoreactivities. In control experiments, total recovery of VIP(1-28) added to spinal cord tissues immediately prior to extraction was 71% of added VIP(1-28), and for VIP(22-28) recovery was 76% of added peptide.
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(means of two independent estimations assayed in triplicate).

**Gel filtration chromatography**

Extracts of rat spinal cord segments, pooled from 10 rats, were concentrated under vacuum and applied to a 1.0 x 100 cm column of Sephadex G-50 (Superfine), previously equilibrated with 10% (v/v) formic acid x4°C. The column was eluted with the same solvent and 2ml fractions were collected. Entire fractions, or aliquots of each fraction, were dried under vacuum and reconstituted in 0.1 M acetic acid, 0.01 M HCl, in 50% aqueous methanol prior to radioimmunoassay of 5 or 10 μl samples from each fraction (equivalent amounts of solvent were added to control and standard curve tubes).

The elution volumes of rat spinal cord VIP immunoreactive peaks were compared with the elution volumes of aliquotes (up to 40 pmol) of synthetic VIP(1-28), and VIP(22-28) determined by RIA. Bovine serum albumin and CoCl₂ were used as external standards to determine by spectrophotometric estimation excluded volume of the column and the total column volume, respectively.

Control experiments were conducted in which authentic VIP(1-28) was added to one half of a pool of 10 half spinal cords, while vehicle was added to the other. Both halves were extracted separately and the extracts fractionated by gel-filtration. VIP(22-28) immunoreactivity, appearing in the elution positions of either VIP(1-28) or VIP(22-28) as a result of the addition of VIP(1-28) to one half of the spinal cord pool, was calculated by subtraction of the amounts of the endogenous ir-VIP(22-28) appearing at each elution position in the absence of added VIP(1-28).

**High performance liquid chromatography**

Fractions from the gel-filtration column containing peaks of VIP immunoreactivity were dried under vacuum and redissolved in a small volume of 0.1 M acetic acid, 0.01 M HCl, in 50% aqueous methanol prior to injection onto a 30 cm x 3.9 mm μ-Bondapak C18 reversed phase column (Waters Assoc., Milford, MA) preequilibrated with 20% acetonitrile in 13 mM trifluoroacetic acid (TFA) in water.

The pooled fractions were eluted with a linear gradient of 20 to 50% acetonitrile in 13 mM aqueous TFA over a period of 30 min at a flow rate of 1 ml/min. One minute (1 ml) fractions were collected, dried under vacuum, and redissolved in 0.1 M acetic acid containing 0.15 M NaCl and 0.1% Triton X-100 for radioimmunoassay.

Reported retention times were determined from the fractions containing peak concentrations to peptide immunoreactivity.

**Results**

*ir-VIP(22-28) in the rat spinal cord*

Six rat spinal cord segments were investigated: estimation of total ir-VIP(22-28) in the initial extracts suggested a different distribution in the rostro-caudal axis of the rat spinal cord. ir-VIP(22-28) was higher in the dorsal area of the cord than in the ventral area, especially in the lumbo-sacral segment (Table). Statistically significant differences were observed among the spinal segments as indicated in the Table. Dilution curves for the spinal cord extracts paralleled the VIP(22-28) standard curve. Our estimates of tissue content are consistent with the previously reported distribution of ir-VIP in rat CNS (12).

**Gel filtration chromatography**

Extracts of each spinal cord region were pooled and concentrated, then subjected to chromatography on Sephadex G-50. Significant peaks of ir-VIP(22-28) were recovered from each region examined. A major peak (peak I) of immunoreactivity eluted after the void volume in the approximate position of the VIP(1-28) standard (Fig. 1). A second major peak (peak II) eluted in the position of the VIP(22-28) standard. No differences in the elution profiles were observed among the six spinal cord regions.

Control experiments were conducted in order to determine if VIP(1-28) was converted to the peak II peptide(s) during the extraction and fractionation procedures. VIP(1-28) was added to five half spinal cord samples before extraction, and chromatography. It was primarily recovered in the elution position of authentic VIP(1-28).
Fig. 1 Gel-filtration fractionation of extracts of rat spinal cord at cervical dorsal (left top panel) and ventral (right top panel) level; at thoracic dorsal (left middle panel) and ventral (right middle panel) level; and at lumbo-sacral dorsal (left bottom panel) and ventral (right bottom panel) level on Sephadex G50. Extracts of each spinal region from groups of 10-15 rats were pooled and fractionated as described in the Method section. The y-axes indicate the fmol of ir-VIP(22-28) in each fraction. Elution positions of peptide standards or column markers run on the same columns are indicated by the arrows at the top of each panel: a, void volume; b, VIP(1-28); c, VIP(22-28); d, total column volume.
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Fig. 2 Gel-filtration fractionation of extracts of rat spinal cords on Sephadex G50 for studies on recovery of the peptides. Five half spinal cords were homogenated as control (solid line and close symbols); authentic VIP(1-28) was added to the remaining halves, prior to homogenization and extraction (dashed line and open symbols). Elution position of peptide standards are indicated by the arrows at the top of the panel: a, VIP(1-28); b, VIP(22-28).

Only a slight increase in immunoreactivity eluting from the G-50 column in the position of VIP(22-28) was observed, to 7.9% of the added VIP(1-28) (Fig. 2). Since the ir-VIP(22-28) eluting as VIP(22-28) in the five remaining half spinal cords (in absence of added VIP(1-28)), was about 31% of the ir-VIP(22-28) eluting as VIP(1-28), it seems that the VIP(22-28) is present in the extract and is not generated from VIP(1-28) during the analytical procedures. These data on rat spinal cord are also consistent with previous concerning the rat brain and the distribution of VIP-related peptides (9).

HPLC

Further characterization of the ir-VIP(22-28) was obtained by reversed phase HPLC of extracts from the lumbar region of 20 rat spinal cords since this region had the highest level of VIP-related peptides. The elution conditions were selected in order to give the best recovery of the small amounts of immunoreactivity present in the extracts. The fractions containing the peaks of ir-VIP(22-28) corresponding in elution position on the Sephadex G-50 column to VIP(1-28) (peak I) and VIP(22-28) (peak II) were pooled, dried down and divided into two aliquots. An amount of authentic VIP(1-28) (for peak I) or VIP(22-28) (for peak II) was added to one of the aliquots. Then the aliquots of the peak without added peptide, with added peptide standard and standard alone, were chromatographed sequentially. The HPLC elution profile of ir-VIP (22-28) in rat lumbar spinal cord is shown in Figure 3.

The G-50 peak I eluted from the HPLC column as a single peak with a retention time of 20 min, equivalent to that of authentic VIP(1-28) standard run alone or added to the peak extract (Fig. 3, left panel). A more complex result was obtained when the G-50 peak II was characterized. Peak II yielded three major peaks of immunoreactivity, one of which corresponded to the elution position of authentic VIP(22-28) run alone or added to the sample, with a retention time of 13 min (Fig. 3, right panel). The other two peaks of ir-VIP(22-28) eluted with retention times of 6 and 19 min.

Characterization on HPLC of the ir-VIP(22-28) in the extracts from the initial six areas of the rat spinal cords was also carried out. Because of the relative small amount of material eluted from the G-50 column, the peaks collected from the cervical and thoracic regions were injected onto the HPLC and run without added standards. All six samples showed same pattern on HPLC; no differences were found between dorsal and ventral segments. Peak I from the G-50 column consisted of one peak in the elution position of VIP(1-28); peak II from the G-50 yielded three major peaks one of which consisted of VIP(22-28).

Table Ir-VIP(22-28) in the Rat Spinal Cord Segments

<table>
<thead>
<tr>
<th>Tissue</th>
<th>ir-VIP 22-28 (pmol/g tissue)</th>
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<tbody>
<tr>
<td>1 - SC CERV D</td>
<td>2.53 ± 0.20</td>
</tr>
<tr>
<td>2 - SC CERV V</td>
<td>1.28 ± 0.10</td>
</tr>
<tr>
<td>3 - SD THOR D</td>
<td>1.74 ± 0.19</td>
</tr>
<tr>
<td>4 - SC THOR V</td>
<td>0.97 ± 0.08</td>
</tr>
<tr>
<td>5 - SC LUM-SAC D</td>
<td>2.92 ± 0.20</td>
</tr>
<tr>
<td>6 - SC LUM-SAC V</td>
<td>2.05 ± 0.20</td>
</tr>
</tbody>
</table>

Values are means ± SEM of 10-15 rats.
1 vs 2: p < 0.01; 3 vs 4: p < 0.01; 5 vs 6: p < 0.01; 1 vs 3: p < 0.01; 1 vs 5: not significant; 3 vs 5: p < 0.01; 2 vs 4: not significant; 2 vs 6: p < 0.01; 4 vs 6: p < 0.01.
Fig. 3 HPLC fractionation of ir-VIP(22-28) in rat lumbo-sacral spinal cord extracts. Peaks of ir-VIP(22-28) eluting from the gel-filtration columns in the approximate positions of authentic porcine VIP(1-28) (Peak 1, left panel) or VIP(22-28) (Peak 2, right panel) were dried and divided into two equal aliquots. An amount of VIP(1-28) (left panel) or VIP(22-28) (right panel) was added to one of the aliquots as an internal standard. The aliquots were then chromatographed on a reversed phase C18 column as described in the Methods section. Close symbols and solid lines indicate results for samples with no added VIP. Dashed lines and open symbols indicate the samples to which an aliquot of VIP(1-28) or VIP(22-28) was added. Retention times of peptide standards run in the absence of tissue extracts are indicated by arrows: a, VIP(22-28); b, VIP(1-28).

Discussion

These results confirm the presence of vasoactive intestinal polypeptide in the rat spinal cord (1, 13, 14). The VIP immunoreactivity is predominantly located in the dorsal horn; it is less concentrated in the ventral area. The distribution is consistent with that reported earlier (12, 15). The highest concentrations of VIP immunoreactivity were in the lumbo-sacral region of the rat spinal cord.

Chromatographic analysis (sequential gel filtration and reverse phase HPLC) of the VIP immunoreactivity demonstrated the occurrence of different molecular forms of VIP immunoreactivity. Gel filtration of the tissue extracts revealed two major peaks of ir-VIP in all the segments. The first peak corresponds to the elution position of authentic porcine VIP(1-28); the second one corresponds in elution position to the VIP carboxy-terminus fragment VIP(22-28). Experiments in which VIP(1-28) was added to spinal cord samples before extraction demonstrate that the VIP(22-28) immunoreactivity was not generated from VIP(1-28) during extraction procedure.

HPLC was used to analyze the peaks from the gel filtration. In the lumbar region the peak with higher molecular weight consisted exclusively of VIP(1-28), as demonstrated by its coelution with the standard. The peak with the lower molecular weight consisted of three components: the central one corresponded to VIP(22-28) and accounted for 20% of the total immunoreactivity. The other two components are still unidentified. The first of these peaks is probably a short carboxy-terminus fragment of VIP and represents 60% of the entire peak II (16). The third VIP(22-28) immunoreactive component eluted at a time very similar to that of VIP(1-28). Since the material applied to the HPLC column was well separated from VIP(1-28) by Sephadex G50 chromatography, the presence of an immunoreactive component eluting from the HPLC column at about the same time as VIP(1-28) probably reflects the presence of a VIP fragment with similar hydrophobicity to VIP(1-28) under our chromatography conditions. However, it might alternatively indicate that a small amount of VIP(1-28) was present in the G50 peak II.

On the whole, our data on rat spinal cord are consistent with our previous results in the central nervous system (9), exhibiting the same chromatographic patterns and confirming the presence of the VIP(22-28) in the entire rat CNS. The biological significance of VIP(22-28) and other VIP fragments in rat spinal cord is still unknown. This peptide could be generated and stored with VIP in secretory vesicles or generated by extracellular peptidases after the secretion of VIP. One speculation is that it may represent a product of the posttranslational processing ‘in vivo’ of VIP(1-28) at the level of nerve ending, acting as an independent neuropeptide. Alternatively, VIP(22-28) could represent a metabolic product of VIP(1-28) released from the nerve terminal; in this case the measurement of VIP(22-28) tissue levels might provide information on the rate of turnover of the peptide VIP under different conditions (17). The possibility that VIP(22-28) represents an index of VIP activity, having or not a role at CNS level, will be investigated in physiological or pharmacological manipulated conditions.
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References