**Novel Tachykinins From the Brain of the Sea Lamprey, *Petromyzon marinus*, and the Skate, *Raja rhina***

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*Regulatory Peptide Center, Creighton University School of Medicine, Omaha, NE 68178, †Department of Biochemistry and Molecular Biology, University of New Hampshire, Durham, NH 03824, ‡Department of Zoophysiology, University of Goteborg, S400 31 Goteborg, Sweden

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WAUGH, D., S. SOWER, C. BJENNING AND J. M. CONLON. Novel tachykinins from the brain of the sea lamprey, *Petromyzon marinus*, and the skate, *Raja rhina*. **Peptides** 15(1) 155–161, 1994.—Using radioimmunoassays for mammalian tachykinins, peptides with substance P-like immunoreactivity and neurokinin A-like immunoreactivity were identified in an extract of the brain of the longnose skate. *Raja rhina* (elasmobranch) but only a peptide with neurokinin A-like immunoreactivity was identified in the brain of the sea lamprey. *Petromyzon marinus* (agnathan). The primary structure of the skate peptide with substance P-like immunoreactivity (Ala-Lys-Asp-Lys-Phe-Tyr-Gly-Leu-Met-NH₂) shows one amino acid substitution (Phe³ → His) compared with scyllochelin 1, previously isolated from dogfish brain and gut. The skate neurokinin A-related peptide (His-Lys-Leu-Gly-Ser-Phe-Val-Gly-Leu-Met-NH₂) shows two substitutions (Thr⁴ → Leu and Asp⁶ → Gly) compared with mammalian neurokinin A. Although the COOH-terminus of the lamprey tachykinin (Arg-Lys-Pro-Arg-Pro-Lys-Glu-Phe-Val-Gly-Leu-Met-NH₂) resembles neurokinin A, the presence of the strongly conserved Lys/Arg-Pro-Xaa-Pro motif at the NH₂-terminus of the peptide indicates greater structural similarity with substance P. The additional arginine residue at position 1 in the peptide suggests that the lamprey is utilizing a site of posttranslational processing in the tachykinin precursor that is different from the equivalent site in mammalian and other lower vertebrate preprotachykinin(s).

**Table 1**

<table>
<thead>
<tr>
<th>Tachykinin</th>
<th>Substance P</th>
<th>Neurokinin A</th>
<th>Elasmobranch</th>
<th>Agnathan</th>
</tr>
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**METHOD**

**Materials**

Lamprey substance P and skate neurokinin A were synthesized by Multiple Peptide Systems, and their identities were confirmed by automated Edman degradation and amino acid composition analysis. Other synthetic peptides were from Peninsula Laboratories. The collection of whole brain from approximately 2000 specimens of adult sea lamprey has been described previously (20). Whole brain was taken from adult specimens of

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TABLE 1

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Column</th>
<th>Elution Gradient [% acetonitrile/water (v/v)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamproy SP</td>
<td>Vydc C-4</td>
<td>14% → 25% in 30 min</td>
</tr>
<tr>
<td></td>
<td>Vydc phenyl</td>
<td>14% → 28% in 40 min</td>
</tr>
<tr>
<td></td>
<td>Vydc C-18</td>
<td>14% → 28% in 40 min</td>
</tr>
<tr>
<td>Skate NKA</td>
<td>Vydc C-4</td>
<td>10.5% → 31.5% in 50 min</td>
</tr>
<tr>
<td></td>
<td>Vydc phenyl</td>
<td>10.5% → 31.5% in 50 min</td>
</tr>
<tr>
<td></td>
<td>Vydc C-18</td>
<td>14% → 31.5% in 50 min</td>
</tr>
<tr>
<td>Skate</td>
<td>Vydc C-4</td>
<td>0% → 35% in 60 min</td>
</tr>
<tr>
<td>Scyliorhinin</td>
<td>Vydc phenyl</td>
<td>0% → 35% in 60 min</td>
</tr>
<tr>
<td></td>
<td>Vydc C-18</td>
<td>14% → 28% in 40 min</td>
</tr>
<tr>
<td></td>
<td>Vydc C-18</td>
<td>14% → 28% in 40 min</td>
</tr>
</tbody>
</table>

Tissue Extraction

Skate brain (211 g) was extracted by boiling in 0.5 M acetic acid (2000 ml) for 10 min. After centrifugation (1600 × g for 60 min), peptides were isolated from the supernatant by adsorption onto Sep-Pak C-18 cartridges (Waters Associates) as described (4). Bound material was recovered by elution with 70% (v/v) acetonitrile/water and lyophilized. The extraction of lamproy brain (155 g) by the same procedure has been described previously (20).

Radioimmunoassay Procedure

Neurokinin A-like immunoreactivity (NKA-LI) was measured using antiserum NKA-2 directed towards the COOH-terminal region of NKA that requires an α-amidated COOH-terminal methionine residue at the COOH-terminus for binding. The antiserum shows only 0.6% cross-reactivity with substance P and 0.5% cross-reactivity with physalaemin. Substance P-like immunoreactivity (SP-LI) was measured using antiserum P-4 directed against the COOH-terminal region of substance P that also requires a COOH-terminal α-amidated methionine residue for binding. The antiserum shows less than 0.1% cross-reactivity with neurokinin A and 33% cross-reactivity with physalaemin. Full details of the radioimmunoassay procedures, sensitivities

skate of both sexes (n = 89, weight range 0.2–5.6 kg) at Bamfield Marine Station, Vancouver Island, Canada during May and June, 1990. The tissue was immediately frozen and stored at −20°C.

![Graph A](image)

![Graph B](image)

FIG. 1. Reverse-phase HPLC on a semipreparative Vydc C-18 column of (A) an extract of lamproy brain and (B) an extract of skate brain after partial purification by gel permeation chromatography. The fractions denoted by the open bars contained substance P-like immunoreactivity and the fractions denoted by the hatched bars contained neurokinin A-like immunoreactivity. In (B), the heights of the bars do not accurately indicate the relative concentrations of the peptides. The dashed line shows the concentration of acetonitrile in the eluting solvent.
TACHYKININS FROM LAMPREY AND SKATE

FIG. 2. Purification of the lamprey tachykinin by reverse-phase HPLC on (A) Vydac C-4, (B) Vydac phenyl, and (C) Vydac C-18 columns. The bar denotes the peak containing NKA-like immunoreactivity and the arrows show where peak collection began and ended. In (C), the large arrow shows the retention time of the synthetic lamprey tachykinin.

of the assays, and the specificities of the antisera have been provided in a recent review (3).

Purification of the Tachykinins

The lamprey brain extract, after partial purification on SepPak cartridges, was redissolved in 1% (v/v) trifluoroacetic acid (10 ml) and chromatographed on a (2.5 x 100 cm) column of Biogel P-10 (BioRad) equilibrated with 1 M acetic acid at a flow rate of 72 ml/h. Fractions (12 ml) were collected and absorbance was measured at 280 nm. Fractions containing NKA-LI were pooled and the material was pumped at a flow rate of 2 ml/min onto a (1 x 25 cm) Vydac 218TP510 (C-18) reverse-phase HPLC column equilibrated with 0.1% (v/v) trifluoroacetic acid/water. The concentration of acetonitrile in the eluting solvent was raised to 21% (v/v) over 10 min, held at this concentration for 30 min, and raised to 49% over 60 min using linear gradients. Absorbance was measured at 214 and 280 nm and fractions (1 min) were collected. The lamprey tachykinin was purified to apparent homogeneity by successive chromatographies on (250 x 4.6 mm) Vydac 214TP54 (C-4), (250 x 4.6 mm) Vydac 219TP54 phenyl, and (250 x 4.6 mm) Vydac 218TP54 (C-18) columns using the elution conditions shown in Table 1. The flow rate was 1.5 ml/min in all cases. Immunoreactive peaks were identified by radioimmunoassay after appropriate dilution of the samples.

The skate brain extract was subjected to gel permeation chromatography (Biogel P-10) under the same conditions used for the lamprey brain extract. Fractions containing both SP-LI and NKA-LI were pooled and subjected to reverse-phase HPLC on a Vydac 218TP510 column, as described for the lamprey brain extract. Skate scyliorhinin I [peak I in Fig. 1(B)] and skate neurokinin A [peak II in Fig. 1(B)] were purified to apparent homogeneity by further chromatography under the conditions shown in Table 1.

Structural Characterization

The primary structures of the fish tachykinins (approximately 500 pmol) were determined by automated Edman degradation using an Applied Biosystems model 471A sequenator modified for on-line detection of phenylthiohydantoin-coupled amino acids (Pth-Xaa) under gradient elution conditions. Standard operating procedures were used and the detection limit for Pth-Xaa was 1 pmol. Californium-252 plasma desorption mass spectrometry was performed using a BIO-ION Nordic BIN-20K time-of-flight instrument. Spectra were recorded at 16 kV for 106 primary fission events. The accuracy of mass determinations was ± 0.1%.

RESULTS

Purification of the Lamprey Tachykinin

The crude extract of lamprey brain, even at high dilution, contained material that inhibited the binding of [2,125I]iodohistidyl].neurokinin A to an antibody directed against the COOH-terminus of neurokinin A. However, the immunoreactivity in serial dilutions of the extract did not diminish in parallel with the neurokinin A standard in radioimmunoassay. The crude lamprey brain extract did not contain material that inhibited the binding of [125I]Bolton–Hunter-labeled substance P to an antibody directed against the COOH-terminus of substance P. The NKA-LI in the extract, after partial purification on SepPak cartridges, was eluted from a Biogel P-10 gel permeation column as a single peak with approximately the same elution volume as mammalian neurokinin A. As shown in Fig. 1(A), the NKA-LI in the pooled fractions from this peak was eluted from a semipreparative Vydac C-18 in two consecutive fractions (shown by the hatched area). Rechromatography of this material on an analytical Vydac C-4 column [Fig. 2(A)] showed that the NKA-LI was eluted well before the major UV-absorbing peaks. The lamprey tachykinin was purified to near homogeneity by successive chromatographies on analytical Vydac phenyl [Fig. 2(B)] and C-18 [Fig. 2(C)] columns. The final yield of pure peptide was approximately 800 pmol.

Purification of the Skate Tachykinins

The SP-LI and NKA-LI in the skate brain extract, after partial purification on SepPak cartridges, was eluted from the Biogel P-10 column in the same fractions that corresponded to the elution volume of substance P. Neither the SP-LI nor the NKA-LI in serial dilutions of the fractions diminished in parallel with the synthetic substance P and neurokinin A standards in radioimmunoassay. As shown in Fig. 1(B), the SP-LI (peak I) and the NKA-LI (peak II) were completely separated by chroma-
FIG. 3. Purification of skate scyllorhinin I [peak I in Fig. 1(B)] by reverse-phase HPLC on (A) Vydac C-4, (B) Vydac phenyl, and (C) Vydac C-18 columns. The bar denotes the peak containing SP-like immunoreactivity and the arrows show where peak collection began and ended.

tography on a semipreparative Vydac C-18 column. The tachykinin peptide in peak I (subsequently shown to be scyllorhinin I) was purified to near homogeneity by successive chromatographies on analytical Vydac C-4 [Fig. 3(A)], Vydac phenyl [Fig. 3(B)], and Vydac C-18 [Fig. 3(C)] columns. After a final purification on the analytical C-18 column, the peptide was eluted as a sharp symmetrical peak and the final yield of pure material was approximately 1200 pmol. The peptide showed absorbance at 280 nm, indicative of the presence of a tyrosine or tryptophan residue.

The tachykinin peptide in peak II (subsequently shown to be skate neurokinin A) was purified to near homogeneity under
the lampry tachykinin was confirmed by mass spectrometry and chemical synthesis. The observed molecular mass of the peptide was 1437.6 compared with a calculated molecular mass of 1437.7 for the C-terminally α-amidated form of the proposed sequence. The retention time of the synthetic lampry tachykinin on a Vyde C-18 column under the elution conditions shown in Fig. 4(A) was 23.5 min compared with a retention time of 23.5 min for the endogenous peptide.

The amino acid sequence of the skate substance P-related peptide was identical to that of scylorhinin I, previously isolated from dogfish gut (4) and brain (21) except for the substitution (Phe → His) at position 3. The primary structure of the peptide was confirmed by mass spectrometry (observed molecular mass 1207.6; calculated molecular mass of the C-terminally α-amidated form of the peptide 1207.6). The amino acid sequence of the skate neurokinin A-related peptide shows two substitutions (Thr → Leu at position 3 and Asp → Gly at position 4) compared with mammalian neurokinin A. The primary structure of the peptide was confirmed by chemical synthesis. Under the elution conditions shown in Table 1, the retention time on a Vyde C-18 column of the synthetic peptide was 36.6 min compared with 36.6 min for the endogenous peptide.

DISCUSSION

This study presents the first reported amino acid sequence of a tachykinin-related peptide from an agnathan. Its primary structure is compared with tachykinins from other vertebrate classes in Fig. 5. The lampry tachykinin shows structural features that are similar to both neurokinin A and substance P. The presence of a valine residue at position 9 (corresponding to position 7 in neurokinin A) accounts for the reactivity of the lampry peptide towards an antisera directed against the C-terminal region of mammalian neurokinin A (3). However, the N-terminal region of lampry peptide shows stronger structural similarity to mammalian substance P. In common with all other substance P-related peptides yet characterized (Fig. 5), the lampry tachykinin contains the motif Lys/Arg-Pro-Xaa-Pro. A recent study (2) using Chinese hamster ovary cells expressing the cloned rat NK1, NK2, and NK3 receptors has shown that the second proline residue (corresponding to position 4 in substance P) is important in conferring selectivity towards NK1 receptors.

<table>
<thead>
<tr>
<th>Cycle No.</th>
<th>Skate Neurokinin A</th>
<th>Skate Scylorhinin I</th>
<th>Lampry Substance P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>His (70)</td>
<td>Ala (215)</td>
<td>Arg (291)</td>
</tr>
<tr>
<td>2</td>
<td>Lys (214)</td>
<td>Lys (147)</td>
<td>Lys (339)</td>
</tr>
<tr>
<td>3</td>
<td>Leu (275)</td>
<td>His (297)</td>
<td>Pro (219)</td>
</tr>
<tr>
<td>4</td>
<td>Gly (269)</td>
<td>Asp (179)</td>
<td>His (159)</td>
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<tr>
<td>5</td>
<td>Ser (33)</td>
<td>Lys (238)</td>
<td>Pro (147)</td>
</tr>
<tr>
<td>6</td>
<td>Phe (139)</td>
<td>Phe (283)</td>
<td>Lys (144)</td>
</tr>
<tr>
<td>7</td>
<td>Val (40)</td>
<td>Tyr (172)</td>
<td>Glu (106)</td>
</tr>
<tr>
<td>8</td>
<td>Gly (64)</td>
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<td>Leu (8)</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td>Met (1)</td>
</tr>
</tbody>
</table>

The values in parentheses show the yields of amino acid phenylthiohydantoins (pmol).

Structural Characterization

It was possible to determine the amino acid sequence of the lampry and skate tachykinins by automated Edman degradation and the results are shown in Table 2. The primary structure of the conditions shown in Table 1 (chromatograms shown in Fig. 4). After the final purification on an analytical Vyde C-18 column, the peptide was eluted as a sharp symmetrical peak that was devoid of absorbance at 280 nm. The final yield of pure peptide was approximately 500 pmol.

FIG. 4. Purification of skate neurokinin A [peak II in Fig. 1(B)] by reverse-phase HPLC on (A) Vyde C-4, (B) Vyde phenyl, and (C) Vyde C-18 columns. The bar denotes the peak containing NKA-like immunoreactivity and the large arrow in (C) shows the retention time of synthetic [Leu⁴,Gly⁷]neurokinin A.
Substance P
Chicken substance P
Alligator substance P
Ranakinin (frog)
Manatachrylin A (frog)
Trot substance P
Cod substance P
Dogfish substance P
Lamprey substance P
Dogfish scyllorhinin I
Skate scyllorhinin I
Physaeanisin (frog)
Neurokinin A
Frog neurokinin A
Trot/cod neurokinin A
Skate neurokinin A
Carassin

FIG. 5. A comparison of the primary structures of peptides related to substance P, scyllorhinin I, and neurokinin A from species from different vertebrate classes. (-) Denotes residue identity and (<E) denotes a pyroglutamyl residue. The lamprey tachykinin was detected using an antisem to neurokinin A but is grouped with substance P on the basis of structural features in the N-terminal region.

On this basis, the lamprey peptide is classified with substance P in Fig. 5 rather than with neurokinin A.

The identification of a single molecular component with tachykinin-like immunoreactivity in the brain of the sea lamprey contrasts with an earlier study (21) in which at least three distinct tachykinins were identified by radioimmunoassay and HPLC in the central nervous systems of the river lamprey, Lampetra fluviatilis, and the silver lamprey, Ichthyomyzon unicuspis. This discrepancy between the two studies may indicate that there is considerable interspecies variability among the Agnatha or that the sea lamprey contains additional tachykinin peptides that are not recognized by the antisera raised against mammalian substance P or neurokinin A. For example, a peptide with a tachykinin-like ability to contract the guinea pig ileum (ranatachrylin D) was recently isolated from the bullfrog intestine and shown to contain the C-terminal sequence Phe-Tyr-Ala-Pro-Met-NH₂ (13). This peptide would probably not be detected with the antisera used in the present study (3).

The biosynthetic relationship between the different tachykinins in lower vertebrates is unknown. In mammals, nucleotide sequence analysis of cloned cDNAs has shown that substance P and neurokinin A are products of the posttranslational processing of the same biosynthetic precursor (β-and γ-propreotachykinin) [reviewed in (16)]. The predicted amino acid sequence in the region of the precursor containing the substance P sequence indicates that generation of substance P involves the proteolytic cleavage of the Arg-Arg bond in the sequence -Ile-Arg-Ala-Arg-Arg-Pro-Lys-. The enzymes responsible for the conversion of the precursor forms of neuroendocrine peptides to the mature secreted forms in mammalian tissues (subtilisin-related proprotein convertases) have now been structurally characterized [reviewed in (14)]. The presence of an additional arginy1 residue at the N-terminus of lamprey substance P suggests that, in this species, the putative processing enzyme is cleaving at the N-terminal side of the Arg-Lys residues at the N-terminus of the peptide rather than between the basic residues. The importance of proline residues in the neighborhood of dibasic residue processing sites has been pointed out by several authors (15,19).

For example, generation of corticotropin-like intermediate lobe peptide (CLIP) in the dogfish pituitary (15) involves cleavage of the sequence -Lys-Arg-Arg-Pro- in proopiomelanocortin. In this case, however, dogfish CLIP contains the sequence Arg-Pro at its amino-terminus, suggesting that the mechanism of prohormone conversion in the elasmobranch tissue follows the mammalian pattern.

The isolation of a neurokinin A-related peptide from the skate brain was an unexpected finding. A previous study (24) using the same techniques of peptide purification and detection, identified in an extract of the brain of the dogfish, a substance P-related peptide ([Lys]\β[Arg]\γ[Glue] substance P), together with scyllorhinin I, but a neurokinin A-related peptide was not detected. The cyclic tachykinin, scyllorhinin II, previously identified in dogfish (4) and Torpedo (7) intestine using the same antisem to neurokinin A as in the present study, was not detected in either dogfish or skate brain. The data indicate that among the elasmobranchs there is appreciable species and tissue differences in the distribution of the different molecular forms of the tachykinins. As shown in Fig. 5, skate neurokinin A shares with a neurokinin A-related peptide isolated from frog intestine (22) the presence of a leucine residue at position 3. Studies with cell lines expressing the cloned mammalian tachykinins receptors (2) have shown that the aspartic acid residue at position 4 is important in conferring selectivity for the NK₁ receptor. The fish neurokinin A-related peptides lack this acidic residue and it has been shown that the goldfish peptide, carassin (Fig. 5), is a relatively poor agonist at the NK₂ receptor in rat fundus (1).

ACKNOWLEDGEMENTS

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