Scyliorhinin I and II: two novel tachykinins from dogfish gut

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Two peptides with tachykinin-like ability to contract longitudinal muscle from the guinea pig ileum were isolated from the intestine of the common dogfish, *Scyliorhinus caniculus*. The amino acid sequence of scyliorhinin I was established as Ala-Lys-Phe-Asp-Lys-Phe-Tyr-Gly-Leu-Met-NH_2 and this peptide cross-reacted with antisera directed against the C-terminal region fo substance P. The amino acid sequence of scyliorhinin II was established as Ser-Pro-Ser-Asn-Ser-Lys-Cys-Pro-Asp-Gly-Pro-Asp-Cys-Phe-Val-Gly-Leu-Met-NH_2 and this peptide cross-reacted with antisera directed against the C-terminal region of neurokinin A. The mammalian peptides substance P and neurokinin A were absent from the dogfish intestinal tissue.

Scyliorhinin (Dogfish) Substance P Neurokinin A Tachykinin

1. INTRODUCTION

The tachykinins are a family of polypeptides that share a common C-terminal sequence, represented by Phe-X-Gly-Leu-Met-NH_2 and a similar spectrum of biological activities. Physalaemin and uperolein (X = Tyr), phyllomedusin (X = Ile) and kassinin (X = Val) were isolated from the skin of frogs and eledoisin (X = Ile) from a mollusc (review [1]). Recent studies have demonstrated that mammalian nervous tissue contains, in addition to substance P (X = Phe), neurokinin A (X = Val) [2] and neurokinin B (X = Val) [3]. Here, preliminary investigations showed that extracts of the intestine of the common dogfish, *Scyliorhinus caniculus,* produced strong, dose-dependent contractions of longitudinal muscle from the guinea pig ileum, suggesting the possible presence of tachykinin-related peptides. By using regionally specific antisera raised against substance P and neurokinin A in radioimmunoassays [4,5], we have isolated from the extracts two novel tachykinins, termed scyliorhinin I and II. Sequence analysis has shown that the peptides show maximum homology with the amphibian peptides, physalaemin and Glu^2Pro^5-kassinin [6].

2. MATERIALS AND METHODS

2.1. *Tissue extraction*

Intestinal tissue (204 g) from 4 adult dogfishes was homogenized at 4°C with 8 vols ethanol/0.7 M HCl (3:1, v/v) using a Waring blender. The homogenate was stirred overnight at 4°C, centrifuged ($1600 \times g$, 1 h) and the ethanol removed from the supernatant under reduced pressure. After further centrifugation ($20000 \times g$, 1 h), the clear supernatant was pumped at a flow rate of 0.5 ml/min onto 8 Sep-Pak C18 cartridges (Waters Associates) connected in series. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (80:19:1) and the effluent lyophilized.

2.2. *Purification of the peptides*

The intestinal extracts, after Sep-Pak concentration, were redissolved in 1% (v/v) trifluoroacetic

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acid (5 ml) and chromatographed at 4°C on a column (100×2.6 cm) of Biogel P-10 (200–400 mesh) eluted at a flow rate of 20 ml/h with 0.2 M acetic acid. Fractions (3.8 ml) were assayed for substance P-like immunoreactivity (SP-LI) and neurokinin A-like immunoreactivity (NKA-LI) at appropriate dilution. The fractions with SP-LI and NKA-LI were separately pooled, lyophilized and redissolved in 1% (v/v) trifluoroacetic acid (1 ml). After filtration (0.45 μm), the fractions were chromatographed on a semi-preparative Supelcosil LC-18-DB column (250×10 mm) eluted at 30°C and at a flow rate of 2 ml/min with a linear gradient (120 ml) formed from acetonitrile/water/trifluoroacetic acid (21.0:78.9:0.1) and acetonitrile/water/trifluoroacetic acid (35.0:64.9:0.1). Absorbance was monitored at 214 and 280 nm and fractions (2 ml) assayed for SP-LI and NKA-LI. The peptide with SP-LI (scyliorhinin I) was purified to homogeneity by chromatography under isocratic elution conditions on a Supelcosil LC-18-DB column (250×4.6 mm) eluted at 30°C and at a flow rate of 1.5 ml/min with acetonitrole/water/trifluoroacetic acid (25.2:74.7:0.1). The peptide with NKA-LI (scyliorhinin II) was purified to homogeneity on the same column using acetonitrile/water/trifluoroacetic acid (23.8:76.1:0.1) for isocratic elution.

2.3. *Structural analysis*

Amino acid composition was determined using a Durrum D-500 automatic analyser as described [7]. The primary structures of the peptides were determined by automated Edman degradation using an Applied Biosystems model 470 A gas-phase sequencer [8]. The detection limit for PTH-amino acids was 0.5 pmol. Scyliorhinin II was reduced and pyridylethylated according to [9].

2.4. *Analytical methods*

SP-LI was measured by radioimmunoassay using antiserum P4 that is directed towards the C-terminal region of substance P [4]. The antiserum shows approx. 35% cross-reactivity with physalaemin, 14% with uperolein but less than 0.1% with the non-amidated form of substance P, its C-terminal methyl ester and with neurokinin A. SP-LI was also measured using antiserum R-140, directed against the N-terminal region of substance P, that shows full cross-reactivity with the non-amidated form of substance P but no detectable cross-reactivity with other tachykinins [10]. NKA-LI was measured using an antiserum directed against the C-terminal region of neurokinin A that reacts fully with neurokinin A (3–10) fragment but shows $<0.1\%$ reactivity with neurokinin A (1–8) and only 0.4% cross-reactivity with substance P [5]. The ability of the tachykinins to contract the longitudinal muscle of the guinea pig ileum was determined as in [11].

3. RESULTS

3.1. *Tachykinin-like immunoreactivity in gut extracts*

The initial extracts of dogfish gut contained immunoreactivity equivalent to 64 pmol/g substance P measured with the C-terminally directed antiserum, no detectable immunoreactivity using the antiserum directed against the N-terminal region of substance P and immunoreactivity equivalent to 61 pmol/g neurokinin A using the C-terminally directed antiserum to neurokinin A. From these results it is concluded that substance P identical to the mammalian sequence is absent from the dogfish gut.

3.2. *Purification of the peptides*

The elution profile on Biogel P-10 of the gut extracts, after concentration on Sep-Pak cartridges, is shown in fig.1. The SP-LI was eluted from the column as a single peak with K_{av} between 0.73 and 0.89. The NKA-LI was eluted as a single peak with K_{av} between 0.55 and 0.68 (chromatogram not shown). As shown in fig.2, the peptide with SP-LI (scyliorhinin I) was eluted from a semi-preparative reverse-phase HPLC column as a single peak with a retention time between 38 and 39 min (substance P: 45.4 min). The peak of immunoreactivity corresponded to the peak of UV conditions of chromatography, the peptide with NKA-LI (scyliorhinin II) was eluted as a single peak with a retention time between 36 and 37 min (neurokinin A: 34.2 min) and the peak of immunoreactivity corresponded to a prominent peak of UV absorbance (fig.3). Under isocratic elution conditions (chromatograms not shown), scyliorhinin I was eluted as a single peak (retention time 15.8 min) that showed absorbance at 214 and 280 nm, indicating the presence of a tyrosine and/or trypto-

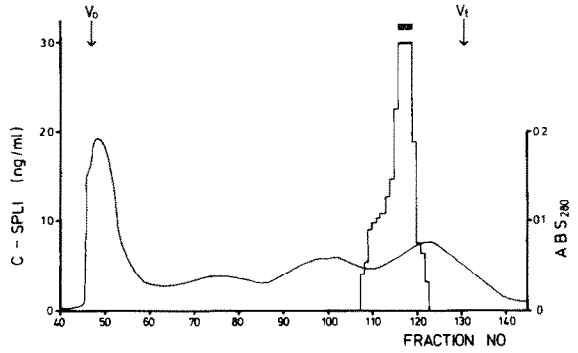

Fig.1. Elution profile on Biogel P-10 of the substance P-like immunoreactivity, measured with a C-terminally directed antiserum, in extracts of dogfish intestine. No detectable immunoreactivity was measured using an antiserum directed towards the N-terminal region of substance P. Neurokinin A-like immunoreactivity was eluted as single peak in fractions 93–104. V_0 and V_t refer to the void volume and total volume of the column and fractions denoted by the hatched bar were subjected to further purification by HPLC.

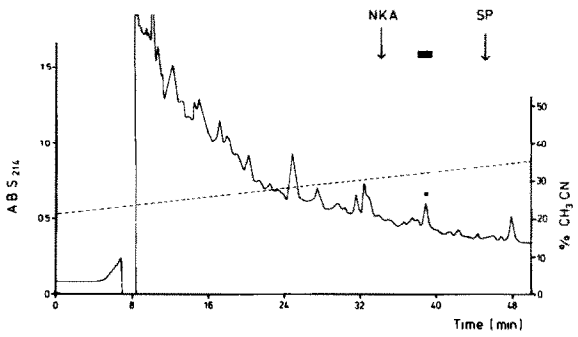

Fig.2. Reverse-phase HPLC on a semi-preparative column of fractions from gel filtration containing substance P-like immunoreactivity (SP-LI). Fractions denoted by the hatched bar contained SP-LI and were further purified by HPLC under isocratic elution conditions. The arrows show the retention times of substance P and neurokinin A; (– – –) concentration of acetonitrile in the elution solvent.

phan residue. The final yield of the peptide was approx. 3 nmol. Scyliorhinin II was eluted under isocratic conditions as a single peak with retention time 18.6 min and was devoid of absorbance at 280 min. The final yield of peptide was approx. 8 nmol.

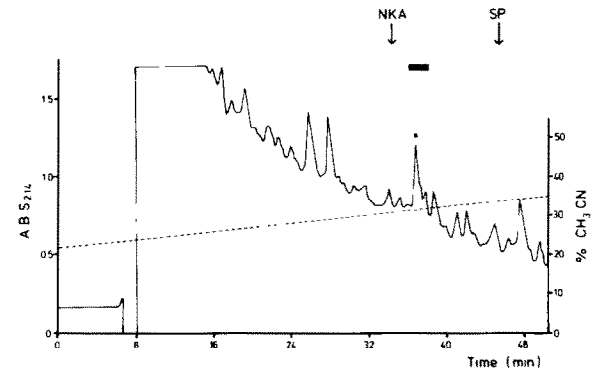

Fig.3. Reverse-phase HPLC on a semi-preparative column of fractions from gel filtration containing neurokinin A-like immunoreactivity (NKA-LI). Fractions denoted by the hatched bar contained NKA-LI and were further purified by HPLC under isocratic elution conditions. Conditions of chromatography were the same as in fig.2 and details are given in the text.

3.3. *Structural analysis*

The results of automated Edman degradation of scyliorhinin I and II (underivatized and pyridylethylated peptide) are shown in table 1. Unambiguous assignment of residues (1–10) of scyliorhinin I was possible. The fall in yield of PTH-amino acids at cycle nos 9 and 10 suggests that the C-terminal methionine residue may be amidated as the Leu-Met-NH_2 and Met-NH_2 would tend to be washed out of the glass fibre disc of the sequencer during the last two degradation cycles. The amount of scyliorhinin I available was insufficient to permit confirmation of the sequence by determination of amino acid composition. Unambiguous assignation of residues (1–17) of scyliorhinin II was possible. The presence of cysteine residues at positions 7 and 13 was demonstrated by subjecting the 4-vinylpyridine-derivatized peptide to Edman degradation. PTH-methionine was detected only in a trace amount at cycle 18 but the presence of a methionine residue in scyliorhinin II was confirmed by amino acid analysis. The composition of the peptide (Asx, 2.94; Ser, 2.66; Pro, 3.40; Gly, 2.28; Val, 0.99; Leu, 1.07; Phe, 0.85; Lys, 1.03; Met, 0.27; Met(oxidized), 0.56) was consistent with the structure determined by Edman degradation but indicated that the methionine residue had been partially oxidized to the sulphoxide derivative during the purification.

Table 1

Automated Edman degradation of scyliorhinin I and II (underivatized and following pyridylethylation of cysteine residues)

Cycle no.	Scyliorhinin I		Scyliorhinin II (underivatized peptide)		Scyliorhinin II (vinylpyridine-treated peptide)	
	PTH amino acid	Yield (pmol)	PTH amino acid	Yield (pmol)	PTH amino acid	Yield (pmol)
1	Ala	1584	Ser	429	Ser	455
2	Lys	2187	Pro	2268	Pro	1122
3	Phe	1648	Ser	203	Ser	216
4	Asp	281	Asn	1991	Asn	1323
5	Lys	1276	Ser	135	Ser	166
6	Phe	1054	Lys	935	Lys	942
7	Tyr	824	–	–	PE-Cys	718
8	Gly	902	Pro	984	Pro	836
9	Leu	508	Asp	371	Asp	323
10	Met	295	Gly	723	Gly	675
11			Pro	506	Pro	588
12			Asp	289	Asp	242
13			–	–	PE-Cys	385
14			Phe	205	Phe	365
15			Val	136	Val	128
16			Gly	109	Gly	77
17			Leu	55	Leu	46
18			(Met)	trace	(Met)	–

PE-Cys, 4-vinylpyridine derivative of cysteine

3.3. *Properties of the scyliorhinins*

As shown in fig.4, serial dilution of purified scyliorhinin I inhibited the binding of ^{125}I-labelled [Tyr^8]substance P to the C-terminally directed antibody but gave rise to a dilution slope that was not parallel to that produced by substance P. This result is consistent with the substitution Tyr for Phe at position 7 of scyliorhinin I and serial dilutions of physalaemin and uperolein also give rise to non-parallel dilution slopes in this radioimmunoassay system. In contrast, serial dilution of purified scyliorhinin II diluted in parallel with neurokinin A using a C-terminally directed antiserum, consistent with the identity of the sequence Phe-Val-Gly-Leu-Met-NH_2 in both peptides.

Sufficient pure material was not available to compare the potencies of the scyliorhinins with the mammalian tachykinins but both scyliorhinin I and II produced an immediate and dose-dependent contraction of the longitudinal muscle of the guinea pig ileum. An amidated C-terminal residue in the tachykinins is an absolute requirement for contractile activity [12] so that the biological properties, as well as the immunochemical properties, of the scyliorhinins strongly suggest that the

Table 2

A comparison of the sequences of the scyliorhinins with tachykinins of amphibian and mammalian origin

Scyliorhinin I	A K F D K F Y G L M
Physalaemin	E A D P N K F Y G L M
Uperolein	E P D P N A F Y G L M
Substance P	R P K P Q Q F F G L M
Scyliorhinin II	S P S N S K C P D G P D C F V G L M
Glu^{2}Pro^5-Kassinin	D V P K P D Q F V G L M
Neurokinin A	H K T D S F V G L M
Neurokinin B	D M H D F F V G L M
Kassinin	D V P K S D Q F V G L M

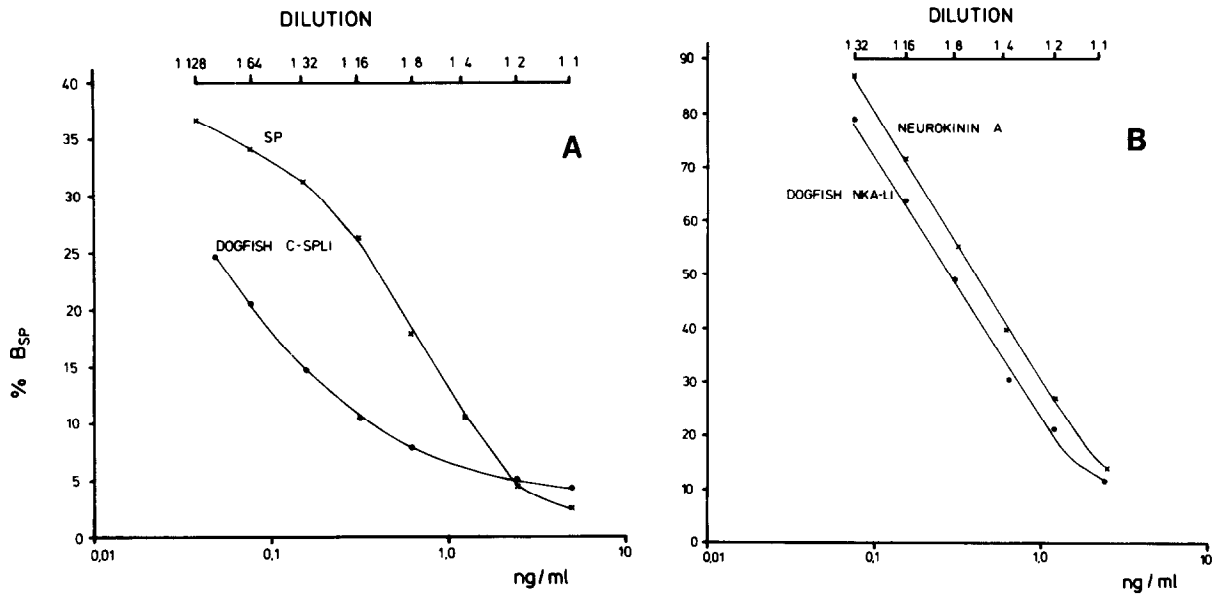

Fig.4. Immunochemical properties of (A) dogfish SP-LI (scyliorhinin I) (●——●) compared with substance P (×—×) in a radioimmunoassay using a C-terminally directed antiserum to substance P and (B) dogfish NKA-LI (scyliorhinin II) (●——●) compared with neurokinin A (×—×) in a radioimmunoassay using a C-terminally directed antiserum to neurokinin A. B_{sp} represents total binding minus binding in the absence of antiserum.

C-terminus of both peptides is amidated. Chemical confirmation of this fact is, however, still required.

4. DISCUSSION

This report has demonstrated that the mammalian peptides, substance P and neurokinin A, are absent from the gut of the common dogfish. The tissue contains, however, two novel peptides with tachykinin-like immunoreactivity and bioactivity. The structures of these peptides, scyliorhinin I and II, are compared with previously characterized tachykinins in table 2. Maximum homology occurs between scyliorhinin I and the amphibian peptide, physalaemin (7 residues) and between scyliorhinin II and the peptide Glu^2Pro^5-kassinin (7 residues), isolated from the skin of the African frog *Hylambates maculatus* [6]. *S. caniculus,* an elasmobranchian cartilaginous fish, diverged from the line of evolution leading to mammals approx. 350 million years ago. If the scyliorhinins and the mammalian tachykinins are derived from the same ancestral tachykinin molecule, this study has demonstrated that evolutionary pressure has operated only to conserve the C-terminal regions of the molecules that are believed to interact with the tachykinin receptors [12].

The presence of tachykinin-like immunoreactivity in the gastointestinal tract and brain of fishes has been demonstrated previously. Consistent with the present report, extracts of the nervous system of skate and mackerel caused a displacement of radiolabelled substance P tracer from a C-terminally directed antibody that was not parallel to the substance P standard curve [13]. Using immunohistochemical techniques, the presence of a substance P-related antigen was demonstrated in nerves of the gut wall of the spiny dogfish, *Squalus acanthias* [14] and in cells of the mid-gut epithelium of the teleostean fish, *Pelmatochromis pulcher* [15]. The sequence Phe-X-Gly-Leu-Met-NH_2 is, however, phylogenetically much older than the fishes. Substance P-like immunoreactivity has been detected in the neural ganglion of the sea squirt, *Ciona intestinalis* (a Urochordate) [16], in nerve cells within the basal disk and tentacles of the Hydra (a coelenterate) [17] and in a giant

neuron of the garden snail, *Helix aspersa* (a mollusc) [18]. The gut and brain of the Atlantic hagfish, *Myxine glutinosa* (a cyclostome), contains both SP-LI and NKA-LI in molecular forms that are distinct from the mammalian peptides (J.M. Conlon and S. Falkmer, unpublished).

In bovine brain, the syntheses of substance P and neurokinin A are directed by the same mRNA [19] and co-localization of the peptides in a population of rat primary sensory neurones has been demonstrated [20]. It is not possible on the basis of this study to conclude that scyliorhinin I and II are derived from the same biosynthetic precursor. Further immunohistochemical work is underway, however, to determine whether the peptides are localised in the same or different populations of nerves.

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