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NEUROPEPTIDE F: PRIMARY STRUCTURE FROM THE TUBELLARIAN, *ARTIOPOSTHIA TRIANGULATA*

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Abstract—1. A neuropeptide exhibiting vertebrate pancreatic polypeptide immunoreactivity has been isolated and sequenced from extracts of the terrestrial turbellarian, *Artioposthia triangulata*.

2. This neuropeptide, designated neuropeptide F, consists of 36 amino acid residues terminating in a phenylalaninamide.

3. The full primary structure was established as: KVVHLRPRSSFSSEDEYQIYLRNVSKYIQLYGR-PRF.NH_2. The molecular mass, deduced from this sequence, was 4433 Da.

4. This neuropeptide exhibits C-terminal homology with neuropeptide F (*Moniezia expansa*) and with the vertebrate neuropeptide Y/pancreatic polypeptide superfamily of which it may represent a phylogenetic precursor.

INTRODUCTION

Neuropeptide F (NPF), a novel 39-residue peptide terminating in a phenylalaninamide, was recently isolated from the tapeworm, *Moniezia expansa*, using a C-terminally directed pancreatic polypeptide (PP) antiserum (Maule *et al.*, 1991). A comparison of the C-terminal regions of amphibian and reptilian PPs (McKay *et al.*, 1990a) with that of NPF, revealed that the C-terminal tetrapeptide amides (RPRF-NH_2) are identical. In addition, the tyrosyl residue positioned 10 amino acids from the C-terminus, is present in all known PP sequences (Maule *et al.*, 1991).

Previous detailed immunocytochemical investigations, employing the C-terminal PP antiserum used in the present study, have demonstrated the widespread distribution of PP-immunoreactivity throughout both central and peripheral nervous systems of numerous parasitic worms (Magee *et al.*, 1989; Maule *et al.*, 1990; McKay *et al.*, 1990b, 1991; Skuce *et al.*, 1990) and the earthworm, *Lumbricus terrestris* (Curry *et al.*, 1989). Radioimmunological investigations, in conjunction with detailed chromatographic analyses of the PP-immunoreactive neuropeptide present in extracts of the fish-gill parasitic worm, *Diclidophora merlangi*, indicated the presence of a single molecular form (*Maule et al.*, 1989a) which co-eluted with synthetic bovine PP following gel permeation chromatography.

The phylum Platyhelminthes contains both free-living and parasitic species which represent the simplest living organisms exhibiting bilateral symmetry with cephalization and centralization of neurones. The ancestral flatworms are generally accepted as the progenitors of most metazoan life representing a strategic phylogenetic stage in metazoan evolution (Barnes *et al.*, 1988). As a previous study (Maule *et al.*, 1991) has determined the primary structure of the PP-immunoreactive neuropeptide (neuropeptide F) from a parasitic species, the present investigation examines the analogous neuropeptide from a species which is free-living.

Preliminary immunocytochemical investigation of the terrestrial tubellarian, *Artioposthia triangulata* (Tricladida: Terricula), demonstrated the presence of extensive neuronal PP-immunoreactivity. *A. triangulata*, which is indigenous to New Zealand, was first recorded in Northern Ireland in the early 1960s (Willis and Edwards, 1977) and has subsequently become well-established throughout the province (Blackshaw, 1990). As single specimens can grow up to 15 cm in length and weigh 2 g, sufficient worm tissue was accessible to facilitate the isolation and sequencing of the PP-immunoreactive neuropeptide.

MATERIALS AND METHODS

Peptide isolation

Specimens of *A. triangulata* ($N = 50$, wet wt 45 g) were collected locally, snap-frozen in liquid nitrogen and stored at −20°C prior to extraction. Frozen worms were thawed in extraction medium (ethanol:0.7 M HCl; 3:1 v/v; 8 ml/g tissue) and manually homogenized in a Teflon/glass homogenizer. The resulting homogenate was stirred overnight at 4°C and then centrifuged at 3000 *g* for 1 hr to remove particulate matter. The ethanol in the supernatant was removed under reduced pressure and the remaining solution was acidified with trifluoroacetic acid (TFA) to a final concentration of 0.1% (v/v). Peptides in this solution were concentrated by pumping through a series of 10 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA) at a flow rate of 10 ml/hr and bound peptides were eluted with acetonitrile. The eluant was then lyophilized. The lyophilized peptide concentrate was reconstituted in 2 ml of 2 M acetic acid and subjected to gel permeation chromatography on a 90 × 1.6 cm column of Sephadex G-50 (medium) (for details, see Maule *et al.*, 1989b). Aliquots of chromatographic fractions (10 μl) were diluted 1:100 in assay buffer

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(40 mM sodium phosphate buffer, pH 7.2, containing 140 mM sodium chloride and 0.2% (w/v) bovine serum albumin) and subjected to C-terminal specific PP radioimmunoassay using antiserum PP 221 (Maule *et al.*, 1991). Pancreatic polypeptide immunoreactivity in gel permeation chromatographic fractions was purified to homogeneity using reverse phase HPLC by employing the following columns in sequence: (1) Partisil 10 ODS-3, C-18, 1 × 60 cm (Whatman, Kent, U.K.); flow rate 3 ml/min. (2) Supelcosil LC-308, C-8, 0.46 × 25 cm (Supelco Inc., Bellfonte, PA); flow rate 1.5 ml/min. (3) Supelcosil LC-3DP, diphenyl, 0.46 × 25 cm; flow rate 1.5 ml/min. The sequential purification of the PP-immunoreactive neuropeptide was monitored by radioimmunoassay of chromatographic fractions and column effluents were monitored spectrophotometrically at 214 nm. Gradients were formed from TFA/water to TFA/water/acetonitrile and are indicated on the respective chromatograms illustrated in Fig. 2(a–c).

Structural analyses

Approximately 50 pmol of purified neuropeptide was subjected to ^{252}Cf-plasma desorption mass spectroscopy using a BioIon 20K time-of-flight instrument. The sample was dissolved in 0.1% (v/v) aqueous TFA and applied to a nitrocellulose-covered target which was spin-dried and micro-rinsed. Spectra were recorded at 16 kV for 10^6 primary fission events.

Approximately 250 pmol of neuropeptide was subjected to automated Edman degradation using an Applied Biosystems 470A gas-phase sequencer. This established the sequence of the first 34 residues with three exceptions and indicated that lysyl residues were present at positions 1 and 26. On the basis of this information, a further 250 pmol of neuropeptide was incubated with 4 pmol of *Armillaria mellea* protease (specific for X-lys peptide bonds) dissolved in 1 μl of water, for 22 hr at 37°C. The reaction mixture was applied directly to the sequencer without fractionation.

Immunocytochemistry

Whole specimens of *A. triangulata* were flat-fixed in 4% (w/v) paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) for 4 hr at 4°C and washed (24 hr) in 5% (w/v) sucrose in PBS prior to cryoprotection in 30% (w/v) sucrose in PBS. The primary antiserum employed was PP 221. Longitudinal, lateral and dorso-ventral cryostat sections (30 μm) of whole specimens and segments were incubated in the primary antiserum (1:200) for 24 hr at 4°C and washed in PBS. Immunostaining was visualised using the indirect immunofluorescence technique (Coons *et al.*, 1955) and the tissue sections were viewed using a MRC 500 confocal scanning laser microscope (Bio-Rad Lasersharp, Abingdon, Oxon, U.K.). Details of the applications of this equipment in studies of invertebrate neurobiology have been reviewed by Johnston *et al.* (1990). Immunocytochemical controls included (1) omission of primary antiserum, (2) substitution of the primary antiserum with non-immune rabbit serum and (3) the liquid preadsorption of the primary antiserum with bovine PP. Two additional controls were employed to eliminate any possible non-specific ionic binding of the primary antiserum: poly-L-lysine (3000 Da) was included at a concentration of 2 mg/ml in the primary antiserum (Scopsi *et al.*, 1986) and, following incubation with the primary antiserum, sections were washed for 1 hr in phosphate-buffered 0.5 M saline (Grube, 1980).

RESULTS

Peptide isolation

Pancreatic polypeptide radioimmunoassay of the crude extract measured 157.5 μg equivalents of immunoreactivity. Similar analysis of crude extracts of 5 separate worms confirmed this striking abundance, detecting a concentration of 3.5 + 0.17 μg equivalents (mean + SEM) of PP-immunoreactivity per gram wet weight of worm tissue. A single immunoreactive molecular form was resolved by gel permeation chromatography and this co-eluted with bovine PP standard (Fig. 1). Sequential reverse-phase HPLC of this material likewise resolved a single immunoreactive peptide in each case (Fig. 2a–c). Approximately 17 μg of neuropeptide was purified to homogeneity representing 10.8% of that present in the crude extract. This apparently low recovery was due to the fact that only peak immunoreactive fractions were re-applied during sequential chromatographic runs. Serial dilutions of PP-immunoreactive fractions at each stage of the purification procedure indicated that in radioimmunoassay, the worm neuropeptide diluted in parallel with bovine PP standards. The molar absorbance of the purified neuropeptide compared favorably with the amount detected by radioimmunoassay indicating that this neuropeptide cross-reacted fully with the PP antiserum employed.

Structural analyses

Plasma desorption mass spectroscopy detected a single predominant ion with a corrected molecular mass of 4440 Da.

Initial gas-phase sequencing of the intact neuropeptide established the sequence of the first 34 residues with three exceptions (Table 1). No PTH-amino acid was detected on cycles 25, 32 and 33. The *Armillaria mellea* protease digest, which was sequenced without prior fractionation, produced the sequences of two fragments corresponding to residues 1–25 and 26–36 of the intact neuropeptide (Table 2). The computed molecular mass, deduced from this entire sequence, was 4433 Da which is in agreement with that obtained by mass spectroscopy. The complete cross-reactivity of the PP antiserum with the worm neuropeptide indicated the presence of an amidated C-terminal residue as this antiserum cross-reacts poorly (<0.01%) with the C-terminal

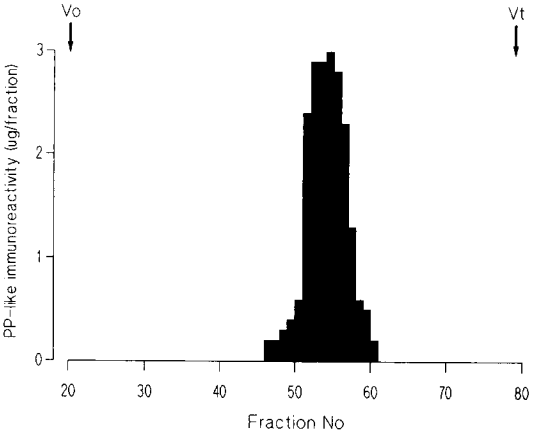

Fig. 1. Gel permeation chromatogram (Sephadex G-50) of PP-immunoreactivity in an extract of *A. triangulata*. The void volume (V_o) and total volume (V_t) of the chromatographic column are indicated.

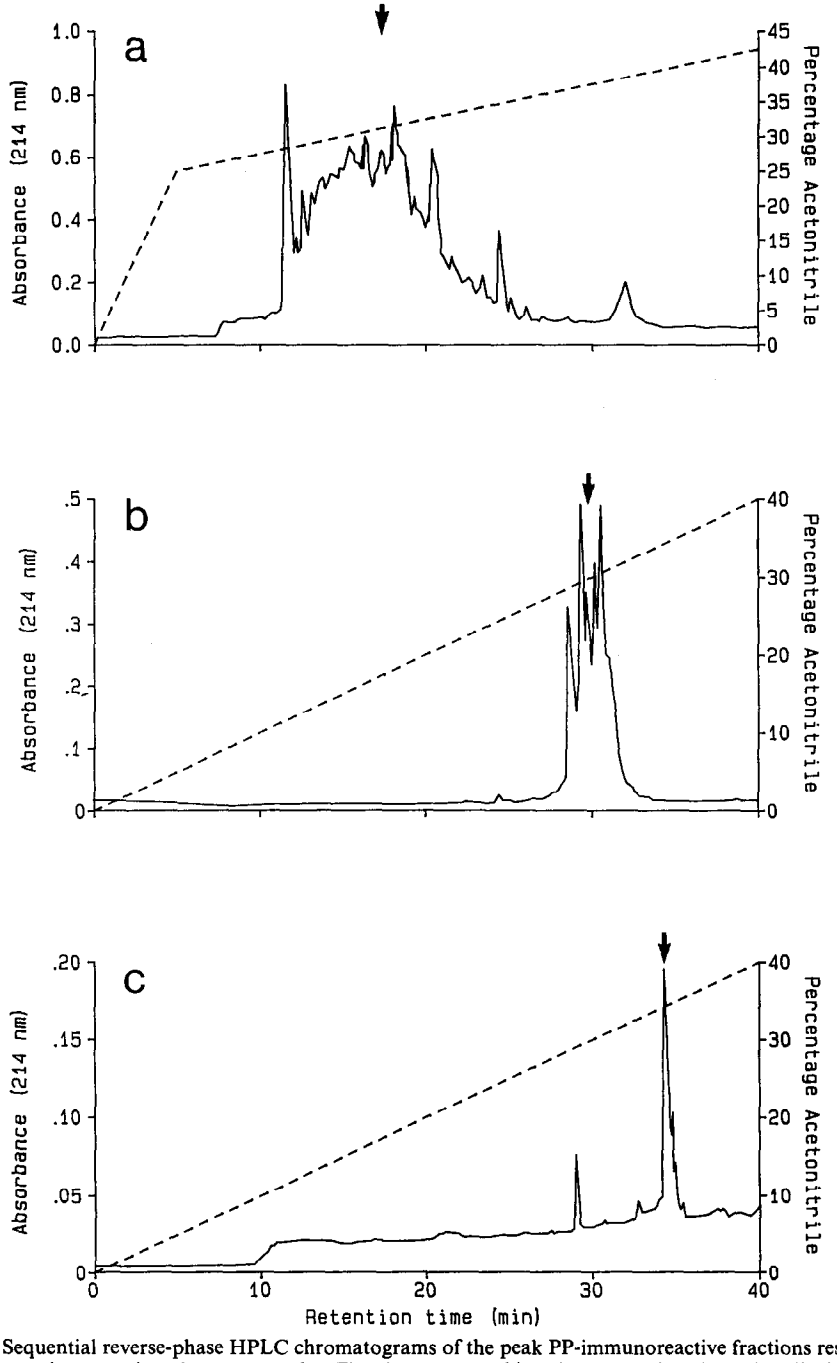

Fig. 2. Sequential reverse-phase HPLC chromatograms of the peak PP-immunoreactive fractions resolved following gel permeation chromatography. The chromatographic columns employed are described in the text. The retention time of PP-immunoreactivity is indicated in each chromatogram and this corresponds to a single discrete peak of absorbance in the final fractionation (c).

hexapeptide free acid of PP. The full primary structure of neuropeptide F obtained from *A. triangulata* is compared with the original neuropeptide F (*Moniezia expansa*) in Fig. 3.

Immunocytochemistry

Intense PP-like immunoreactivity was evident throughout the central and peripheral nervous system of *A. triangulata* (Fig. 4). PP-like immunoreactivity was evident in two lateral ventral nerve cords which extended from the anterior to posterior (Fig. 4a). Immunoreactivity was evident in cell bodies and numerous fibres present in the muscular pharynx (Fig. 4b). These fibres also extended into the extrusible region of the pharynx (Fig. 4c). Preincubation of the antisera with bovine PP (400 ng/ml) abolished immunostaining. No immunostaining was evident with the omission of primary antiserum or the substitution of non-immune rabbit serum. The two additional controls to abolish any non-specific

Fig. 3. Comparison of the complete primary structures of *A. triangulata* (a) and *M. expansa* (b) neuropeptide F. The homologous residues (arrow) and conservatively-substituted residue (circle) are indicated.

interaction of the primary antisera did not diminish the intensity of immunostaining.

DISCUSSION

Recently, the complete primary structure of a novel parasitic platyhelminth regulatory peptide (neuropeptide F) of 39 amino acid residues terminating in a phenylalaninamide, was reported (Maule *et al.*, 1991). The identification and isolation of this neuropeptide utilised an antiserum generated to the highly-conserved C-terminal hexapeptide amide of mammalian pancreatic polypeptide (PP). In the present study, the full primary structure of the analogous neuropeptide has been determined in the free-living terrestrial turbellarian, *Artioposthia triangulata*, utilising the same antiserum. Comparison of the primary structure of both neuropeptides revealed a high degree of homology in their C-terminal regions with 9 of the 17 C-terminal residues in identical positions. The C-terminal pentapeptide amide (GRPRF-NH_2) is completely conserved, as is the tyrosyl residue positioned 10 residues from the C-terminus and the tripeptide sequence (YLR) 15–17 residues from the C-terminus. The leucyl and isoleucyl residues positioned 7 residues from the C-terminus of NPF (*A. triangulata*) and NPF (M. expansa), respectively, represent a conservative substitution. The larger number of residues in NPF (*M. expansa*) relative to NPF (*A. triangulata*) and all known members of the vertebrate NPY superfamily, may have resulted from genetic insertion of deletion processes which are phenomena previously encountered during phylogenetic studies of regulatory peptides. It is also evident that strong evolutionary constraints have been placed on the aromatic residues at positions 10 and 17 from the C-terminus, the arginyl residues at positions 2 and 4 from the C-terminus and the amidated aromatic residue present at the C-terminus, as these residues are highly-conserved in all members of the vertebrate NPY/PP superfamily and invertebrate NPF family so far sequenced.

Previous reports have suggested that PP-immunoreactivity present in invertebrate nervous systems may be due to cross-reactivity of the PP antisera employed with FMRF-NH_2 and related peptides (Dockray and Williams, 1983). However, with the determination of the primary structures of two novel invertebrate neuropeptides exhibiting obvious structural homology in the C-terminus to the vertebrate NPY/PP superfamily and terminating in an RF-NH_2, this suggestion is no longer tenable. The relative abundance of NPF compared to levels of FMRF-NH_2 in invertebrates (Stretton *et al.*, 1992) would tend to suggest that FMRF-NH_2 antisera are most likely also cross-reactive with NPF. Consequently, future immunocytochemical studies of FMRF-NH_2 localisation in the invertebrate nervous systems will

Table 1. Automated Edman degradation of intact neuropeptide F (*A. triangulata*)

Cycle No.	PTH-a.a.	Yield (pmol)
1	Lys	188
2	Val	200
3	Val	227
4	His	61
5	Leu	212
6	Arg	125
7	Pro	187
8	Arg	120
9	Ser	42
10	Ser	41
11	Phe	97
12	Ser	24
13	Ser	25
14	Glu	45
15	Asp	38
16	Glu	41
17	Tyr	38
18	Gln	35
19	Ile	34
20	Tyr	38
21	Leu	27
22	Arg	33
23	Asn	36
24	Val	18
25	X	—
26	Lys	9
27	Tyr	12
28	Ile	6
29	Gln	8
30	Leu	4
31	Tyr	9
32	X	—
33	X	—
34	Pro	5

Table 2. Automated Edman degradation of the *Armillaria mellea* protease digest of neuropeptide F (*A. triangulata*)

Cycle No.	PTH-a.a	Yield (pmol)	PTH-a.a	Yield (pmol)
1	Lys	131	Lys	131
2	Val	102	Tyr	78
3	Val	120	Ile	82
4	His	65	Gln	75
5	Leu	115	Leu	115
6	Arg	73	Tyr	57
7	Pro	120	Gly	44
8	Arg	50	Arg	50
9	Ser	22	Pro	56
10	Ser	20	Arg	45
11	Phe	30	Phe	30
12	Ser	14		
13	Ser	15		
14	Glu	30		
15	Asp	26		
16	Glu	26		
17	Tyr	18		
18	Gln	17		
19	Ile	14		
20	Tyr	13		
21	Leu	trace		
22	Arg	11		
23	Asn	11		
24	Val	6		
25	Ser	trace		
Fragment		1–25		26–36

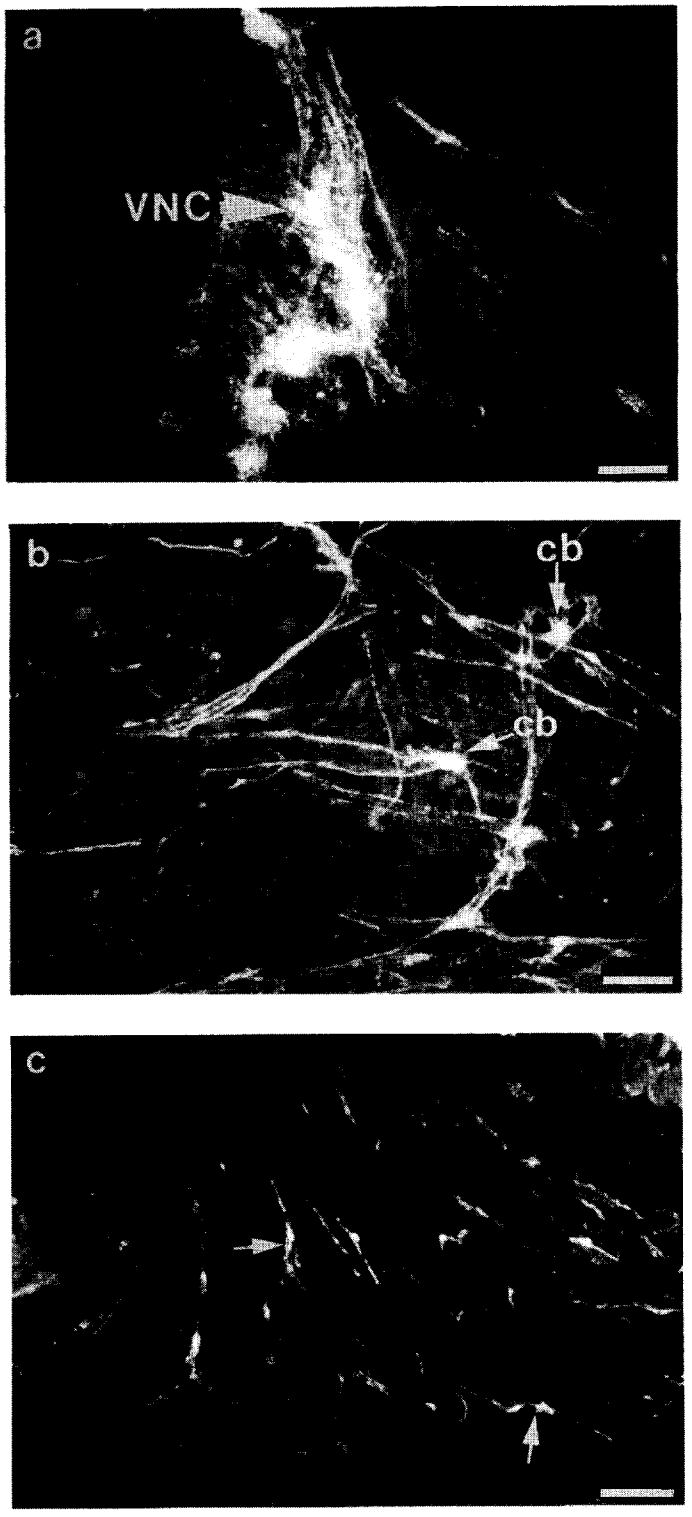

Fig. 4. Confocal scanning laser microscope image of intense PP-immunoreactivity in a lateral ventral nerve cord (VNC) (a) [× 150; Bar + 60 μm], cell bodies (cb) and fibres in the muscular pharynx (b) [× 300; Bar = 30 μm] and in fibres (arrows) in the muscular extrusible pharynx (c) [× 150; Bar = 60 μm].

have to be viewed with caution. *A. triangulata* and *M. expansa* are representative species of the phylum Platyhelminthes that have evolved from a common ancestral flatworm and have adopted radically different strategies for survival. However, over this long evolutionary period, certain amino acid residues within the structure of their respective NPF molecules have been completely conserved; this is indicative of a conserved biological function. The extensive network of neurones and fibres containing NPF would

suggest that this neuropeptide performs an important neurotransmitter function and, due to the extensive innervation observed within the extrusable muscular pharynx, possibly regulates muscular activity. NPF represents a novel invertebrate regulatory peptide family which may constitute the phylogenetic precursors of the vertebrate NPY/PP superfamily.

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