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FMRFamide-LIKE IMMUNOREACTIVITY IN THE CRAYFISH NERVOUS SYSTEM

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Summary

FMRFamide-like immunoreactivity (FLI) was detected in the nervous system of the crayfish *Procambarus clarkii* using an antiserum that recognizes extended RFamide peptides. Immunocytochemistry revealed FLI in neuronal somata, axons and varicose processes within the central nervous system. In the periphery, plexuses of immunoreactive varicosities were present in the pericardial organs (POs), in thoracic roots and on the hindgut. The hindgut plexus arose from 3–5 axons leaving the sixth abdominal ganglion (A6) *via* the intestinal nerve. The presence of FLI in these locations was confirmed by radioimmunoassay. In contrast, no FLI was detected in motor axons innervating exoskeletal muscles of the abdomen.

The POs contained by far the largest amount of FLI of all tissues examined. The immunoreactive material was partially characterized by extraction and separation on two consecutive reversed-phase high performance liquid chromatography (RP-HPLC) columns. The largest amount of immunoreactivity on the second column co-eluted with a synthetic peptide, SDRNFLRFamide (F_2), previously identified as one of two or more FMRFamide-related peptides contained in lobster POs. The immunoreactive fractions and peptide F_2 elicited similar effects on isolated crayfish hearts; all increased the rate and amplitude of spontaneous cardiac contractions. As with the immunoreactivity, the highest level of bioactivity was contained in the fraction that co-eluted with F_2.

The results suggest that FMRFamide-related peptides act as neurohormones in crayfish and are likely to play roles in controlling circulation and defecation.

Introduction

Since the discovery of the neuropeptide FMRFamide in the bivalve mollusc *Macrocallista nimbosa* (Price and Greenberg, 1977), FMRFamide-related pep-

Key words: neurohormone, FMRFamide, crustacean, neuropeptide, pericardial organs, *Procambarus clarkii*.

tides (FaRPs) have been reported in the nervous systems of several invertebrates (Lehman and Price, 1987; Li and Calabrese, 1987; Marder *et al.* 1987; Myers and Evans, 1985; Robb *et al.* 1989; Watson *et al.* 1984). In lobsters, FMRFamide-like immunoactive material is widely distributed throughout the nervous system (Kobierski *et al.* 1987). Immunoreactivity was particularly concentrated in the pericardial organs (POs), which are known to release neurosecretory products that modify cardiac and neuromuscular functions (Cooke and Sullivan, 1982; Kravitz *et al.* 1980). Immunoreactivity was also found in two other potential sites for neurosecretory release: the second thoracic roots and the connective tissue sheath surrounding the nerve cord (Kobierski *et al.* 1987). Although some of the immunoreactive cell bodies may correspond to motoneurones, no immunoreactivity was observed in any of the motor roots. It has been suggested (Kobierski *et al.* 1987) that FaRPs may act as neurohormones.

Extracts of lobster POs were found to contain several FMRFamide-like immunoreactive peptides (Trimmer *et al.* 1987), two of which have yielded the sequences TNRNFLRFamide (F_1, Kravitz *et al.* 1987) and SDRNFLRFamide (F_2). These two peptides are reported to elicit cardiac and neuromuscular excitation in lobster (Kravitz *et al.* 1987) and have been shown to increase synaptic transmitter release and nerve-evoked tension in a phasic neuromuscular system of crayfish (Mercier *et al.* 1990). The peptides also co-elute with material extracted from locust neurohaemal organs, which enhance synaptic transmission and tension in the locust extensor tibiae muscle (Schiebe *et al.* 1990).

The initial goal of the present study was to determine whether FMRFamide-like immunoreactivity (FLI) is present in the crayfish nervous system. Since peptides F_1 and F_2 modulate synaptic transmission and contraction of deep abdominal extensor muscles of crayfish (Mercier *et al.* 1990), we were particularly interested in determining whether FLI would be present in neurosecretory structures such as the POs, and whether it may also be present in any of the motor nerves. We report that FLI is present throughout the crayfish central nervous system (CNS) and is particularly concentrated in the POs. The POs contain material that co-elutes with F_1 and F_2 on two reversed-phase high performance liquid chromatography (RP-HPLC) systems and which has similar bioactivity to F_2 when tested on the isolated crayfish heart. In addition, an FMRFamide-like immunoreactive plexus on the hindgut, not previously reported in any crustacean, was observed.

Materials and methods

Animals

Crayfish (*Procambarus clarkii* Girard) were obtained commercially and were kept in aerated freshwater tanks at 14–15°C on a mixed vegetable diet. Immunohistochemistry and heart bioassays were performed using crayfish that were about 5 cm long and weighed approximately 3 g. Radioimmunoassays (RIA) and HPLC were performed on material from larger animals, approximately 8–11 cm long and weighing about 30–50 g.

Immunohistochemistry

Immunohistochemistry was performed on tissues dissected in physiological saline (see heart bioassay, below). Tissues were fixed in 2 % paraformaldehyde in Millonig's buffer, and whole mounts were processed for FMRFamide-like immunoreactivity using standard procedures described previously (Lange *et al.* 1988). We used a commercially available antiserum generated in rabbit immunised with FMRFamide conjugated to bovine thyroglobulin (Incstar, Stillwater, MN). The antiserum was used at a dilution of 1:1000 and was incubated with tissues for 48–72 h at 4 °C. Following washes at 21 °C, the tissues were incubated in 1:200 fluorescein isothiocyanate (FITC)-labelled sheep anti-rabbit IgG (Daymar Laboratories, Toronto, Ontario) for 18 h at 4 °C and were viewed, after washing, in 5 % *n*-propyl gallate in glycerol, pH 7.3, using epi-illumination fluorescence microscopy.

Extraction and HPLC

Tissues were pooled following dissection in physiological saline and were placed in 500 μl of methanol/acetic acid/water (90:9:1) over ice. Following sonication, the suspension was centrifuged at 8800 *g* for 5 min. The supernatant was removed to another tube and evaporated to dryness using a Speed-Vac concentrator (Savant, Farmingdale, NY). In some instances the pellet was redissolved in 100 μl of 0.5 mol l^{-1} NaOH for subsequent protein analysis using the BioRad protein assay kit with gamma globulin as standard. The dried supernatants were either prepared for RIA (see below) or redissolved in 1–3 ml of water and run (three times) through a C18-containing Sep-Pak cartridge (Waters Associates, Milford, MA) that had previously been equilibrated sequentially with 8 ml of methanol, 8 ml of water, 8 ml of 0.1 % trifluoroacetic acid (TFA) and 2 ml of 0.1 % TFA containing 10 μg of bovine serum albumin (BSA). After washing the cartridge with 8 ml of 0.1 % TFA, samples were eluted using 5 ml of methanol containing 0.1 % TFA and were then evaporated to dryness. Samples were redissolved in chromatography solvent for HPLC separation. Extracts were chromatographed sequentially on two different systems. All separations were done at ambient temperature using an SP8700 solvent delivery system (Spectra Physics, San Jose, CA) and a Retriever II fraction collector (ISCO Ltd, Lincoln, NE). System 1 used a Brownlee RP-18 Spheri-5 column (4.6 mm×25 cm) with a linear concentration gradient of 9 %–66 % acetonitrile containing 0.1 % TFA at 1 ml min^{-1}. The gradient was run over 38 min, beginning 2 min after sample injection. Selected fractions from system 1, showing FLI measured by RIA (see below), were evaporated to dryness, redissolved in HPLC solvent and run on system 2. System 2 consisted of a linear concentration gradient of 18 %–60 % acetonitrile in 0.1 % TFA at 1 ml min^{-1} using a Brownlee Phenyl Spheri-5 column (4.6 mm×25 cm). The gradient was run over 60 min starting 2 min after sample injection. Fractions were again tested for FLI using RIA. For bioassay, chromatographed fractions were evaporated to dryness, redissolved in physiological saline, and bioassayed (see below).

HPLC grade reagents (American Burdick and Jackson, Canlab, Toronto, Ontario) were used throughout.

Radioimmunoassay

The radioimmunoassay (RIA) was initially established using iodinated YFMRFamide (specific activity 438–990 Ci mol^{-1}) purchased from Peninsula Laboratories, Inc. (Belmont, CA). For this study, however, we made our own iodinated YFMRFamide by a typical Chloramine T method. Essentially, 1 mCi of ^{125}I (as 10 μl, Amersham, Arlington Heights, IL), 10 μl of 0.25 mol l^{-1} phosphate buffer (pH 7.5), 5 μg of YFMRFamide (as 10 μl in 0.05 mol l^{-1} phosphate buffer) and 5 μg of Chloramine T (as 10 μl in 0.05 mol l^{-1} phosphate buffer) were mixed, and the reaction was stopped after 30 s with 10 μg of sodium metabisulphite (as 10 μl in 0.05 mol l^{-1} phosphate buffer). The reaction mixture was diluted with 1 ml of 0.25 mol l^{-1} phosphate buffer and was passed through a C18 Sep-Pak cartridge, which had previously been equilibrated with 8 ml of methanol, 8 ml of water, 8 ml of 0.1 % TFA and 2 ml of 0.1 % TFA containing 10 μg of BSA. The Sep-Pak cartridge was washed with 10 ml of 0.05 mol l^{-1} phosphate buffer, and then the peptide was eluted with 5 ml of methanol containing 0.1 % TFA. The tracer was stored at −20 °C in methanol or diluted with RIA buffer.

For RIA, the same antiserum used for immunohistochemistry was diluted with 0.1 mol l^{-1} phosphate buffer (pH 7.4) containing 0.3 % NaCl, 0.1 % BSA and 0.01 % NaN_3 (RIA buffer) and was used at a final dilution of between 1:150 000 and 1:200 000. Iodinated YFMRFamide was diluted in RIA buffer to obtain 7000–10 000 cts min^{-1} for 100 μl. Diluted antiserum (100 μl) and [^{125}I]YFMRFamide (100 μl) were combined with the sample (100 μl in RIA buffer) or with a standard solution containing known quantities of FMRFamide (100 μl in RIA buffer) in the range 5–1000 fmol. The tubes were vortexed and incubated at 4 °C for 15–20 h. Unbound peptide was precipitated by the addition of 250 μl of dextran-coated charcoal (0.4 g of Norit A activated charcoal and 0.04 g of dextran in 50 ml of RIA buffer). The suspension was vortexed, left to stand for 3 min, and centrifuged at 8800 *g* for 6 min; 400 μl of supernatant was removed and counted for 1 min using a Gamma counter.

Heart bioassay

The dorsal carapace, containing the heart and pericardium, was dissected out and was pinned to a Sylgard-lined dish with the ventral side up. The pericardial membrane was severed and pinned at each side to allow the bathing fluid access to the heart. The recording chamber, which had a volume of 0.5 ml, was perfused with physiological saline (van Harreveld, 1936) at a rate of 3.0 ml min^{-1} using a peristaltic pump. The saline had the following constituents (in mmol l^{-1}): Na^+, 205; Cl^-, 232; K^+, 5.3; Ca^{2+}, 13.5; Mg^{2+}, 2.5; Hepes, 5.0 (pH 7.4). The temperature was maintained at 15 °C. Contractions were recorded by connecting the sternal artery (at the posterior end of the heart) to a tension transducer using

two insect pins hooked to the artery at one end and glued to the transducer at the other end.

RP-HPLC fractions of extracts from the PO were dried using a Speed-Vac, re-dissolved in saline and assayed for bioactivity as follows. The perfusion was stopped and, after a few minutes, 0.05 ml fractions (or dilutions) were pipetted into the recording chamber. Bioactive fractions were removed by perfusion. The amount of bioactivity was quantified by applying various doses of F_2 in the identical manner to the same preparations and constructing standard curves. In other experiments, the dose–response characteristics of the effects of F_2 on heart rate and peak tension were determined by changing the perfusate to a solution containing a known concentration of the peptide and perfusing the chamber continuously for several minutes.

Chemicals

Peptides F_1 and F_2 were synthesized by Dr D. McCormick, Mayo Foundation, Rochester, Minnesota, YGGFLRFamide, YGGFMRFamide, FMRFamide and FLRFamide were provided through Dr M. Schiebe (see Schiebe *et al.* 1990), cholecystokinin octapeptide, leucomyosuppressin (LMS) and SchistoFLRFamide were obtained from Peninsula Laboratories, Inc. (Belmont, CA). Antibodies to FMRFamide were obtained from Incstar (Stillwater, MN). All other chemicals were obtained from Sigma Chemical Co. (St Louis, MO), unless stated otherwise.

Results

Immunohistochemistry

Staining with fluorescently labelled secondary antibody indicated that FMRFamide-like immunoreactive material is widely distributed in the crayfish nervous system. Within the CNS, cell bodies, axons, fine branches and varicosities were distinguishable. Outside the CNS, immunoreactivity was found in four sites: the thoracic roots, the POs, the intestinal nerve and the hindgut. In control experiments, immunoreactivity was abolished or greatly reduced in all these areas by pre-incubating the anti-FMRFamide antiserum with 100–200 $\mu g\,ml^{-1}$ (0.172–0.344 $mmol\,l^{-1}$) FMRFamide. Pre-incubation with 50 $\mu g\,ml^{-1}$ bovine pancreatic polypeptide had no observable effect on the staining. Immunohistochemistry was carried out on a total of seven animals, aside from controls. We did not attempt to make a detailed map of the immunoreactive processes for the entire nervous system or of differences that might occur between males and females. The following brief description highlights the main features observed, with specific ganglia chosen as examples.

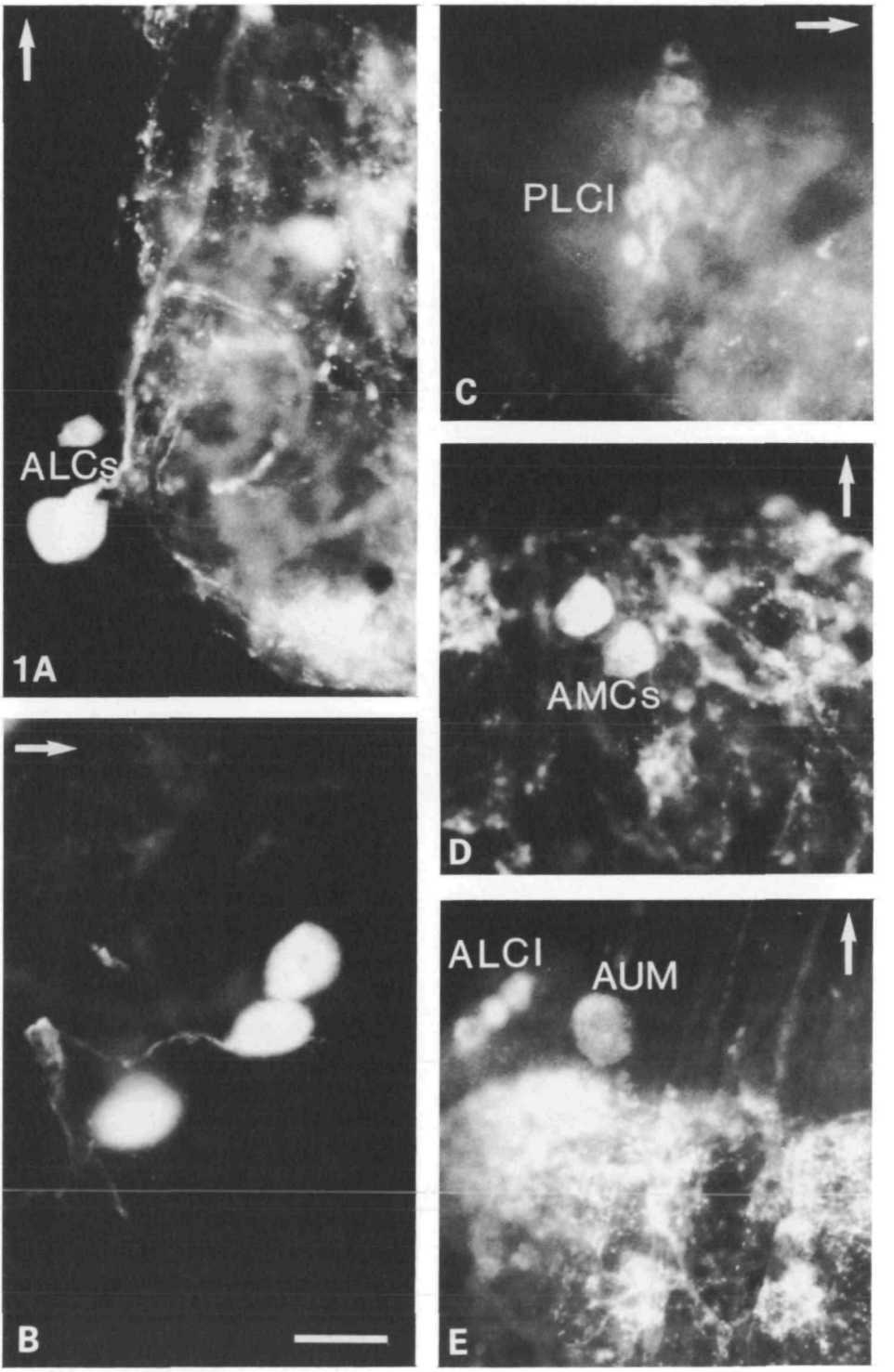
Immunoreactive cell bodies were visible in the suboesophageal ganglion (SOG), in the circumoesophageal ganglia (COG), in each of the five thoracic ganglia (T1–T5) and in each of the six abdominal ganglia (A1–A6). The cell bodies were often present in clusters, particularly in the COG, SOG and thoracic ganglia. One conspicuous group consisted of 3–4 brightly stained cell bodies with large

diameters (approximately 100 μm) in the anterior lateral portion of the SOG (Figs 1A,B, 2A), referred to as an anterior lateral cluster (ALCl). In the thoracic ganglia, clusters of smaller somata, sometimes faintly stained, were observed in the anterior lateral and posterior lateral regions (e.g. Figs 1C, 2B). In the abdominal ganglia, cell bodies were usually present in pairs or in small clusters of only a few cells (e.g. Fig. 1D,E). One notable exception was a large, unpaired cell body (AUM) in the anterior region of ganglia A2–A6 (e.g. Figs 1E, 2C,D). Estimates of the number of immunoreactive somata were 24 and 54, respectively, for the COG and the SOG, and ranged from 20 to 40 for individual thoracic ganglia and from 4 to 12 for the abdominal ganglia. *Camera lucida* drawings of selected ganglia (SOG, T5, A4 and A6) are shown in Fig. 2.

A network of varicose processes was present in many of the ganglia. Such processes were prominent in the SOG (Fig. 3C) and in the abdominal ganglia (e.g. Figs 1E, 2C,D). Fine branches with associated varicosities were also present in the connectives between ganglia (Fig. 3B,D). One striking feature in the CNS was the presence of two brightly labelled medial axons (Fig. 3A) that extended through almost the entire length of the nerve cord. These axons, which were near the dorsal surface, could be traced from the suboesophageal ganglion to the sixth abdominal ganglion. Small varicosities, which occurred along the length of the two axons, gave them a beaded appearance. A similar pair of immunoreactive axons was reported in the lobster (Kobierski *et al.* 1987) and may contribute to the processes that are associated with the connective tissue sheath surrounding the cord. De-sheathing the dorsal surface of the crayfish nerve cord, however, did not remove or disrupt these two axons, suggesting that they lie below the sheath. The location of the cell bodies of these two axons is unknown. In addition, 2–4 immunoreactive axons were present on the ventral side of the nerve cord and were less intensely stained than the two dorsal axons.

Large, brightly stained varicosities were found throughout the POs (Fig. 4B) and in the proximal portions of some of the roots leaving the thoracic nerve cord (Fig. 4E). This suggests the presence of neurosecretory terminals containing FMRFamide-related material. Immunoreactivity was also found in axons leading to the POs *via* the thoracic nerves (Fig. 4A). The intestinal nerve, which leaves the

Fig. 1. Examples of immunoreactive cell bodies in the crayfish central nervous system (CNS). (A) Two brightly stained cell bodies (ALCs) in the anterior lateral portion of the suboesophageal ganglion (SOG) are shown in saggital view; one of these sends an immunoreactive axon into the neuropile region, where there are numerous varicosities. These two cells are part of a small cluster of 3–4 somata (ALCl), the remainder of which were not in the focal plane. (B) In a different preparation, a dorsal view of the anterior lateral portion of the SOG shows three brightly stained somata near the ventral surface; these comprise the ALCl. (C) A cluster of somata (PLCl) in the left posterior lateral region of T5 contains both brightly and faintly labelled neurones. Immunoreactive cell bodies and varicosities are also shown in the anterior regions of ganglia A6 (D) and A4 (E). ALCs, anterior lateral cells; ALCl, anterior lateral cluster; AMCs, anterior medial cells; AUM, anterior unpaired medial cell; PLCl, posterior lateral cluster. Arrows indicate anterior. Scale bar, 100 μm.


ALCs
1A
PLCI
C
AMCs
D
ALCI
AUM
E
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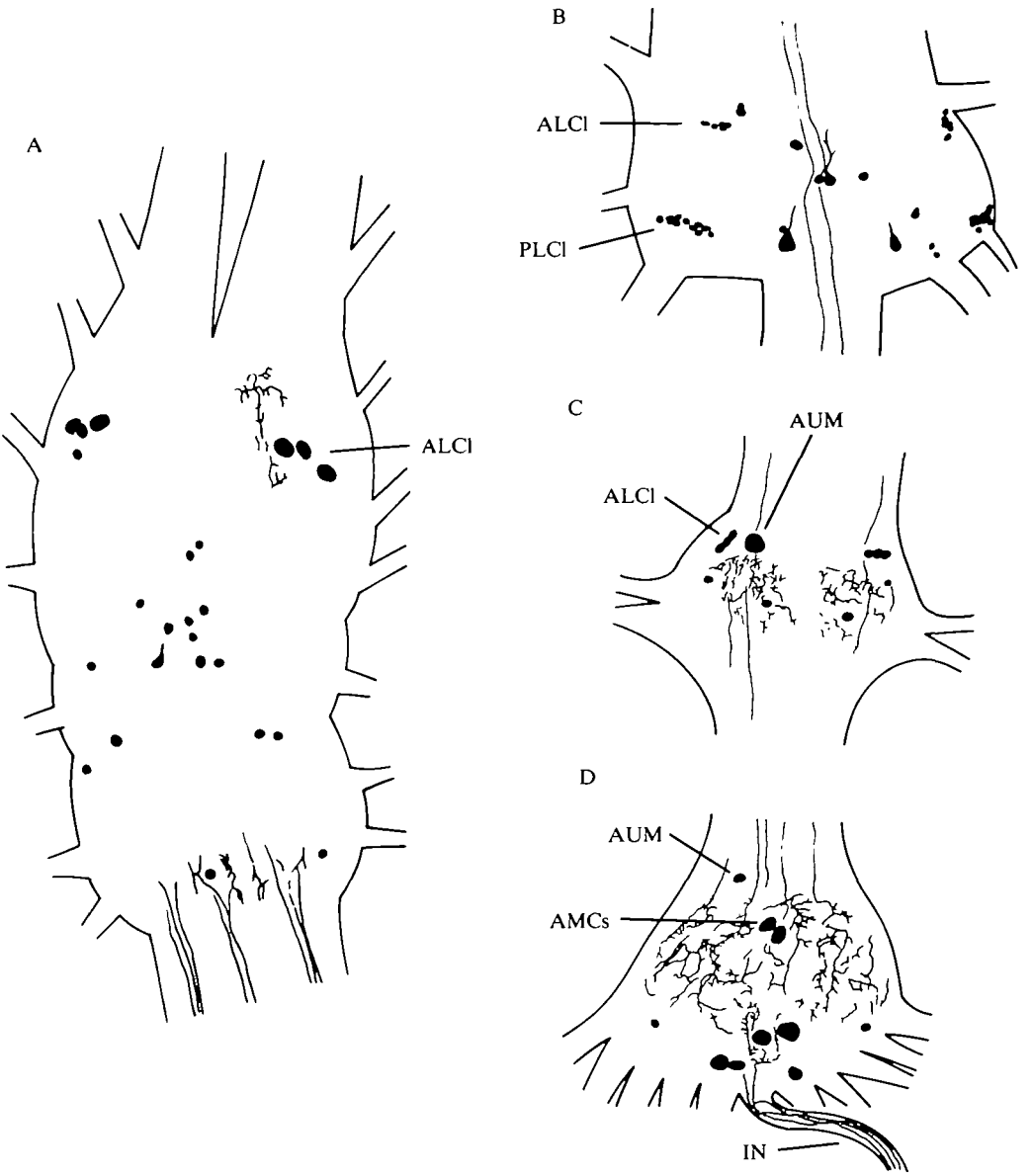

Fig. 2. *Camera lucida* drawings of the SOG (A) and of ganglia T5 (B), A4 (C) and A6 (D), showing the locations of the cell bodies and clusters shown in Fig. 1. In each ganglion, the immunoreactive cell bodies were present near the ventral surface, and most of the varicosities were observed near the dorsal surface. Dorsal varicosities were omitted from drawings of the SOG and T5 for simplicity. IN, intestinal nerve; other abbreviations as in Fig. 1.

sixth abdominal ganglion (A6) and innervates the hindgut (Muramoto, 1977), contained 5–6 immunoreactive axons (Fig. 4C). These could be traced from A6 to a plexus of fine branches and varicosities on the hindgut (Fig. 4D). The plexus

extended posteriorly towards the anus and anteriorly as far as the first abdominal segment.

In some preparations, fine processes with varicosities were associated with the first and second roots leaving the abdominal nerve cord. Such processes, however, were restricted to the proximal portions and did not extend very far along the length of the roots. None of the axons entering or leaving the first, second or third roots of any of the abdominal ganglia was immunoreactive. No immunoreactivity was present in any of the axons entering the deep or superficial abdominal extensor muscles or in any axons entering the deep or superficial abdominal flexor muscles, and no immunoreactivity was associated with any of the muscles themselves.

Radioimmunoassay

The presence of FMRFamide-like immunoreactive material in the central and peripheral nervous systems was confirmed using RIA (Table 1). The amount of activity present was expressed in 'FMRFamide-equivalents'; that is, as the amount of FMRFamide that would displace the same amount of labelled tracer ([^{125}I]YFMRFamide) as that displaced by the tissue extract (see Materials and

Table 1. *Distribution of FMRFamide-like immunoreactivity (FLI) determined by radioimmunoassay*

	FMRFamide-equivalents	
Tissue	(pmol tissue^{-1})	(pmol mg^{-1} protein)
Pericardial organ	63.56±6.86 (5)	2098.0±107 (5)
Heart	0.23±0.09 (3)	0.32±0.13 (3)
Hindgut	3.08±0.54 (5)	3.41±0.78 (4)
Intestinal nerve	0.64 (1)	–
Extensor nerves	0.03±0.002 (3)	–
Extensor muscles	0.03±0.002 (3)	–
Brain	17.38±1.89 (3)	28.93±3.14 (3)
Circumoesophageal ganglion	3.02±0.55 (3)	12.05±2.17 (3)
Suboesophageal ganglion	18.07±4.22 (3)	46.34±10.8 (3)
T1	2.14±0.84 (3)	9.68±3.75 (3)
T2	1.78±0.15 (3)	10.42±0.89 (3)
T3	1.59±0.31 (3)	8.84±1.63 (3)
T4	1.00±0.11 (3)	7.15±0.83 (3)
T5	1.17±0.17 (3)	8.39±1.22 (3)
A1	0.44±0.12 (3)	8.88±2.33 (3)
A2	0.39±0.03 (3)	6.46±0.59 (3)
A3	0.39±0.01 (3)	7.81±0.21 (3)
A4	0.29±0.04 (3)	7.18±1.08 (3)
A5	0.26±0.05 (3)	6.42±1.31 (3)
A6	1.02±0.10 (3)	20.34±1.97 (3)

Values are means±S.E.M. (*N*).
Thoracic ganglia are numbered T1–T5; abdominal ganglia are numbered A1–A6.

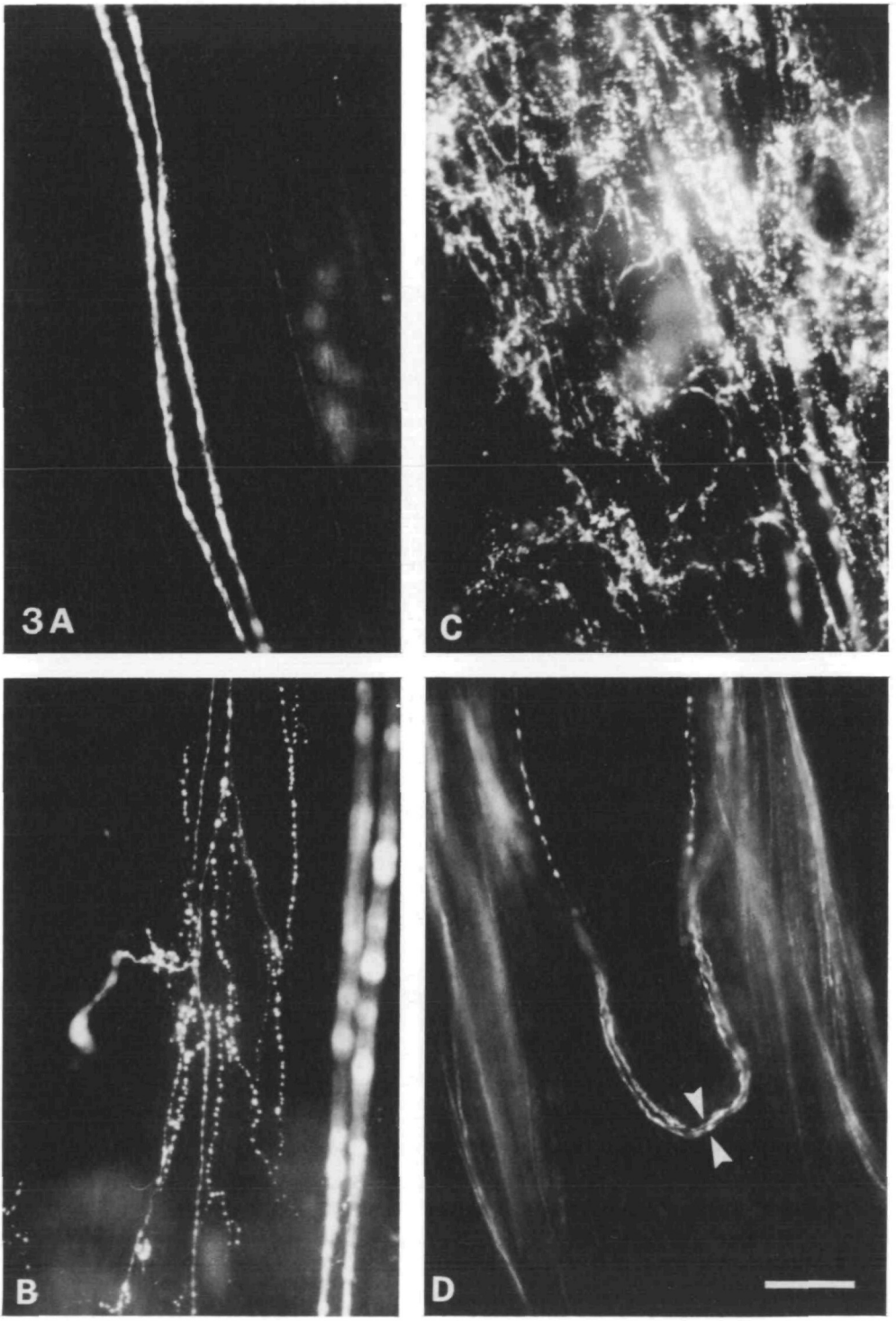
3A
C
B
D

Fig. 3. FMRFamide-like immunoreactivity (FLI) in axons and fine processes. (A) Two brightly stained medial axons, which could be traced from the SOG to A6, are shown in the dorsal part of the nerve cord between T3 and T4. Networks of varicose processes were present near the dorsal surface of the CNS and are shown (B) in the nerve cord between T1 and T2, (C) in the SOG and (D) in the nerve (arrowheads) that joins the circumoesophageal connectives. Scale bar, 100 μm. Anterior is up in all cases.

methods). The antiserum used was specific for extended RFamide peptides and showed equal affinity for FMRFamide, F_1, F_2 and YGGFMRFamide. There was no cross-reactivity with YGGFMRF, with bovine pancreatic polypeptide or with cholecystokinin octapeptide. Every ganglion in the CNS contained significant amounts of activity. Within the CNS, the SOG contained the highest levels of total activity (in FMRFamide-equivalents) and specific activity (in FMRFamide-equivalents mg^{-1} protein). Specific activity was also high in the brain and in ganglion A6 compared to the other ganglia.

Of all structures examined, the POs contained by far the highest levels of total immunoreactivity and of specific activity. We did not attempt to separate the processes contributed to the POs by each individual thoracic nerve, as Kobierski *et al.* (1987) did for the lobster. Instead, one 'PO' in Table 1 represents the pooled contributions from all the thoracic nerves on one side of one animal. Thus, the animals in the present study, which weighed up to 50 g, had an average of about 63.6 pmol of FMRFamide-equivalent activity in the POs on each side, or a total of about 127 pmol of FMRFamide-equivalent immunoreactivity in the POs. Specific activity within the POs (Table 1) was about 45 times that of the SOG, 70 times that of the brain and 100 times that of ganglion A6.

Significant amounts of immunoreactivity were present in the hindgut, and there were measurable amounts in the intestinal nerve that leads to it. In contrast, very little activity was present in the heart, and only background levels were present in the abdominal extensor nerves and in the abdominal extensor muscles (Table 1). These results confirm the presence of FMRFamide-like immunoreactive material in specific tissues and agree with the observed pattern of immunohistochemical staining.

Since the POs contained the most immunoreactivity, and since they are known to release a number of substances that modulate cardiac function, synaptic transmission and muscle tension (Cooke and Sullivan, 1982; Kravitz *et al.* 1980), the remainder of the study focused on characterizing FMRFamide-related material in the POs. It dealt with the question of whether crayfish POs contain material similar to identified peptides, such as F_1 and F_2 of the lobster *Homarus americanus* (Trimmer *et al.* 1987).

Heart bioassay

To aid in characterizing immunoreactive material from the POs, a bioassay was developed, to be used in conjunction with the RIA. Spontaneous contractions were recorded from isolated hearts of small crayfish (see Materials and methods).

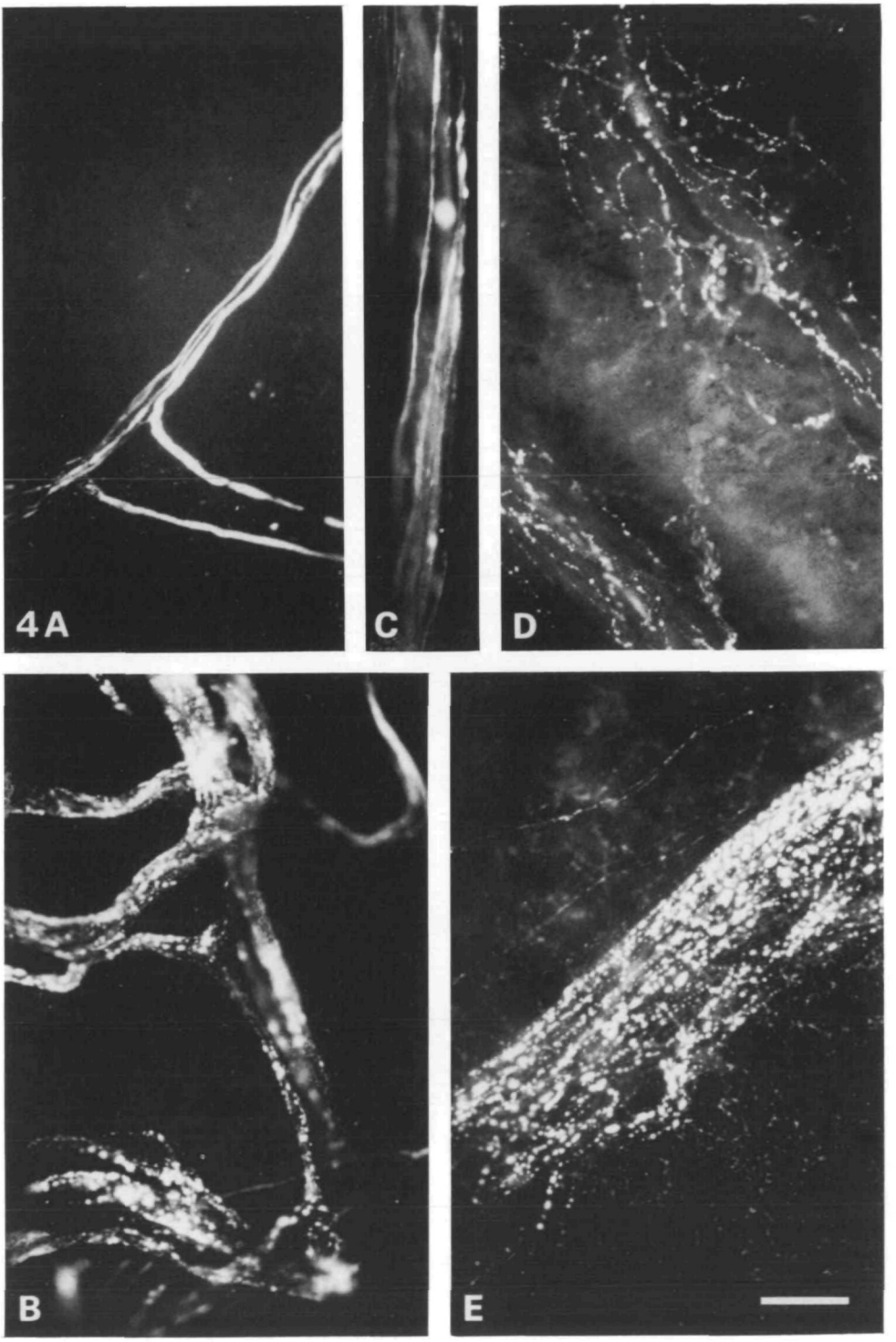
4A
C
D
B
E

Fig. 4. Immunoreactive processes in the peripheral nervous system. In the thorax, at least two large immunoreactive axons in the thoracic segmental nerves (A) could be traced to the POs, where they appeared to give rise to numerous large, bright varicosities (B). In the abdomen, immunoreactive axons were observed in the intestinal nerve (C) and could be traced to a network of varicosities on the hindgut (D). Numerous varicosities were also labelled in some of the roots leaving the thoracic ganglia (E). Scale bar, 100 μm.

Peptide F_2 increased the rate and amplitude of the contractions in a dose-dependent manner (Fig. 5). The threshold concentration for both effects was between 10^{-10} and 10^{-9} mol l^{-1}. However, the effect on amplitude was more pronounced than the effect on rate. The application of 10^{-8} mol l^{-1} F_2 caused a doubling of heart rate and increased the amplitude of contractions three- to fourfold. Since the heart was sensitive to such low doses of F_2, and since the effects could be reversed and repeated by wash-out and re-application of the peptide, F_2 was chosen as a standard for assaying bioactivity in extracts from the POs.

High performance liquid chromatography

Extracts from the POs were fractionated by RP-HPLC using two sequential columns. On the first, a C18 column, 78 % of the immunoreactivity (detected using the RIA) eluted in a broad peak between 22 and 25 min (Fig. 6A). Standard solutions of FLRFamide, F_1, F_2 and YGGFMRFamide all eluted within this time frame. Only background amounts of immunoreactivity appeared at the same elution time as FMRFamide, and only small amounts appeared at times corresponding to leucomyosuppressin (LMS, Holman *et al.* 1986) and SchistoFLRFamide (Robb *et al.* 1989).

The fractions that eluted between 22 and 25 min were pooled and run on a Phenyl column for further purification (Fig. 6B). Approximately 65 % of the activity recovered from the Phenyl column eluted between 29 and 32 min, at times corresponding to F_1, F_2, YGGFMRFamide and YGGFLRFamide. Within this range, the highest level of immunoreactivity (about 37 % of the total) coincided with F_2 (29–30 min), and the next highest value (17 % of the total, which ran at 30–31 min) eluted close to F_1. Collectively, about 25 % of the total FLI available in one PO (64 pmol, see Table 1) was recovered in these two fractions. Significant levels of immunoreactivity also appeared at 33 min and in a pooled fraction from 36 to 44 min.

Fractions from the Phenyl column were dried and resuspended in crayfish saline for bioassay using the isolated heart preparation (Fig. 7). Significant levels of bioactivity were found in the fractions that contained high amounts of immunoreactivity (Fig. 6C). In all cases, the fractions increased both the rate and amplitude of cardiac contractions. As with immunoreactivity, the highest level of bioactivity appeared in the fraction (30–31 min) corresponding to the elution time of F_2. In addition, significant amounts of activity appeared at 29–30 min (coincident with F_1), 33–34 min and from 36 to 44 min. Thus, the POs contained

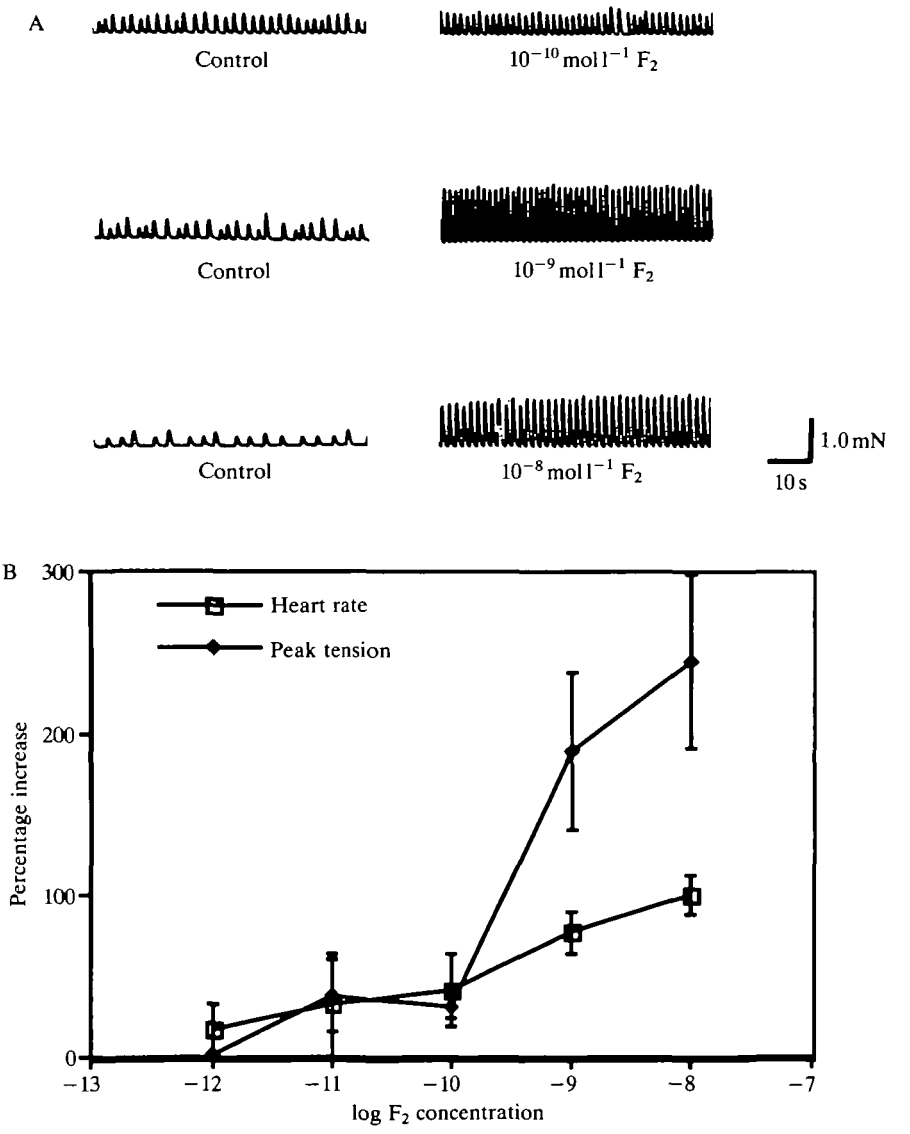

Fig. 5. Effects of lobster peptide F_2 (SDRNFLRF-NH_2, see Trimmer *et al.* 1987) on crayfish heart. (A) Sample chart recordings of cardiac contractions show that F_2 increases the rate and amplitude. (B) A dose–response curve from five preparations (N=5 for each point) shows the threshold for effects on rate and amplitude to be approximately 10^{-10} mol l^{-1}. Error bars in B represent standard errors of the means. F_2 concentration was measured in mol l^{-1}.

FMRFamide-like immunoreactive material that co-elutes with lobster peptides F_2 and F_1 on two different RP-HPLC columns, and this material exhibits bioactivity resembling that of F_2.

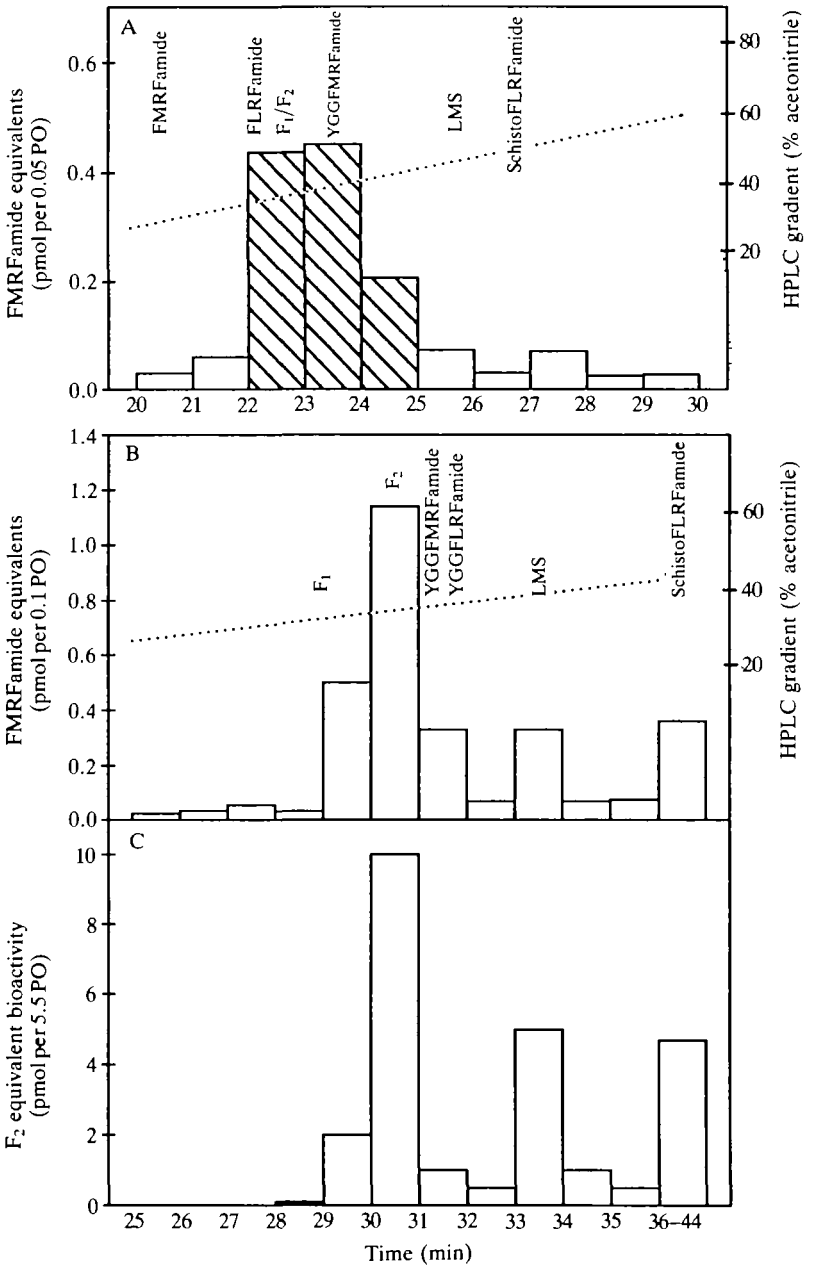

Fig. 6. RP-HPLC of extracts from crayfish pericardial organs (POs). The extracts were first passed through a C18-containing Sep-Pak cartridge, and then chromatographed on an RP-C18 column using a linear acetonitrile gradient in 0.1 % trifluoroacetic acid; fractions were collected at 1 ml min^{-1} and were assayed using radioimmunoassay (RIA) (A). Fractions eluting between 22 and 25 min, which contained approximately 78 % of the recoverable immunoreactivity, were pooled and run on a Phenyl Spheri-5 column at 1 ml min^{-1}. Fractions from the latter column were assayed using RIA (B) and the crayfish heart bioassay (C). See text for details. LMS, leucomyosuppressin.

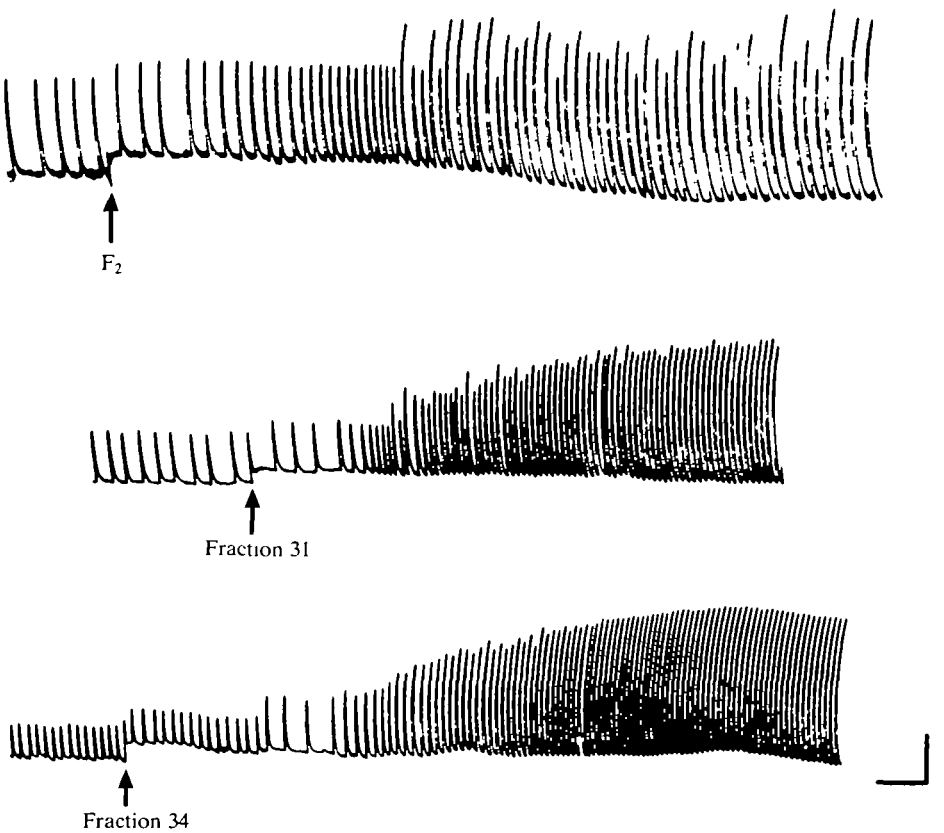

Fig. 7. Bioactivity of peptide F_2 and two fractions from the Phenyl RP-HPLC column. Tension was recorded from an isolated crayfish heart in a 0.5 ml chamber. Arrows indicate the application of 50 μl of saline containing the substances indicated, which caused a shift in baseline tension in all cases, due to the increased volume in the chamber. The response to F_2 was to an applied dose of 50 fmol, which gave the lowest detectable effect. Higher doses of F_2 (not shown) caused a greater increase in tension and more closely resembled the responses shown for the fractions. Fractions 31 and 34 contained 5% and 50%, respectively, of the material extracted from POs and recovered at 30–31 min and 33–34 min from the Phenyl column (Fig. 6). Calibration bars, vertical 0.25 mN top trace, 0.50 mN middle and bottom traces; horizontal, 15 s for all traces.

Discussion

Several observations suggest that FaRPs act as neurohormones in crayfish. First, large immunoreactive varicosities are located within the POs and the thoracic roots, which have been shown to release other neuromodulatory agents into the general circulation (Cooke and Sullivan, 1982; Kravitz *et al.* 1980). Second, the POs contain by far the highest levels of FLI of all the structures examined. Third, immunoreactive varicosities within the nerve cord and in the ganglia are present near the dorsal surface. In the lobster, electron micrographs indicate that such

varicosities (which contain FLI) are associated with the connective tissue sheath (Kobierski *et al.* 1987). This has suggested that FMRFamide-related material may be secreted within the CNS to act on potential targets in the neuropile, a short distance away.

The pattern of immunocytochemical staining is similar to that observed by Kobierski *et al.* (1987) for the lobster. Both organisms have large numbers of immunoreactive somata in the suboesophageal, thoracic and abdominal ganglia, and the cell bodies often appeared in clusters. In both systems there are two immunoreactive axons in the dorsal–medial portion of the nerve cord that run through most of its length, and there are several axons on the ventral surface as well. In addition, both have extensive networks of varicosities in the dorsal surface of the nerve cord, particularly in the SOG. Few, if any, of the motor axons leaving the CNS appear to be immunoreactive. Kobierski *et al.* (1987) reported FLI-containing branches and varicosities in the third roots of ganglia T5 and A1 of the lobster, but it is not clear whether these represented motor axons rather than neurosecretory cells associated with the connective tissue sheath. In the crayfish, none of the motor axons supplying the abdominal skeletal muscles was immunoreactive. Thus, there is no definitive evidence that FaRPs act as cotransmitters at synapses onto crustacean striated muscle.

The pattern of immunocytochemical staining of neurones supplying the hindgut of crayfish is different from that of lobsters. Kobierski *et al.* (1987) observed FLI in a cluster of cell bodies in the posterior region of ganglion A6. This region contains cells whose axons project to the hindgut *via* the intestinal nerves and initiate coordinated contractions of the hindgut during defecation (Winlow and Laverack, 1972). However, no FLI was reported in the intestinal nerves or on the hindgut of the lobster. In crayfish, FLI is present in somata within ganglion A6, in axons running through the intestinal nerve and in a plexus on the hindgut. It is not clear whether these represent species-dependent differences or differences in the specificities of the antibodies used. However, Siwicki and Bishop (1986) reported that the intestinal nerves of lobsters contain proctolin-like immunoreactive axons, while the intestinal nerve of crayfish does not. Thus, it is possible that hindgut motility may be coordinated by the release of proctolin in the lobster and by FaRPs in the crayfish.

The presence of FLI in cells associated with crayfish hindgut adds to a growing body of evidence implicating members of the FMRFamide-related family of peptides in modulating feeding and digestion in arthropods. For example, FLI has been reported in the stomatogastric ganglia of the spiny lobster and the rock crab (Marder *et al.* 1987) and, in the latter, peptide F_2 stimulates the gastric rhythm. In insects, FLI has been observed in the stomatogastric nervous systems (Boer *et al.* 1980), in the midgut (Brown *et al.* 1986) and in varicose processes associated with the hindgut (Tsang, 1990). This suggests that FaRPs are involved in coordinating gastric and intestinal movements.

The amount of FLI in the crayfish nervous system is higher than that reported for lobster. For example, the combined POs on one side of one lobster contained

approximately 10 pmol of FLI (Kobierski *et al.* 1987), compared to 64 pmol for the comparable POs of crayfish (Table 1). However, Kobierski *et al.* (1987) used an antiserum that was more specific for FMRFamide and indicated that their values may underestimate the actual levels of peptides with FLRFamide carboxy terminals by a factor of four. The antiserum used in the present study was equally reactive with FMRFamide, FLRFamide, F_1 and F_2, and this would appear to account for the observed difference.

The POs appear to contain sufficient amounts of FLI to elicit physiological effects on the crayfish heart. The threshold concentration for reliable effects on the rate and amplitude of cardiac contractions by F_2 is about 10^{-10} mol l^{-1}. Although the fluid volume within the pericardial sinus is not known, the total volume of the excised heart, pericardial membrane and epimeral walls, estimated by water displacement, is about 2 ml for a 50 g crayfish (A. J. Mercier, unpublished data). Using this value (which overestimates the pericardial sinus volume) the POs would have to release approximately 0.2 pmol of F_2-equivalent bioactivity to reach the threshold concentration in the pericardial space. The peak fraction from the Phenyl column, representing material that co-elutes with peptide F_2 through two consecutive RP-HPLC columns, contains approximately 11 pmol of FMRFamide-equivalents per PO (Fig. 6B). If all the material in this peak were authentic F_2, the threshold for cardiac effects would be achieved by the release of 0.9 % of the total available in the combined POs of one crayfish. The amount of bioactivity within this peak, however, is only equivalent to about 1.8 pmol of F_2 per PO (Fig. 6C), which would give a value of 3.6 pmol of F_2-equivalent bioactivity in one animal. Based on the bioassay, the POs would have to release approximately 6 % of the material corresponding to this peak to achieve the threshold for cardiac effects.

The reason for the discrepancy between the amount of bioactivity and the amount of FLI in the peak fraction is not known. Since the antiserum used in the present study had equal affinities for F_2, FMRFamide and other RFamide peptides, it might be expected that the amount of F_2-equivalent bioactivity in the RP-HPLC fractions would be comparable to the amount of FLI. The material within the peak fraction could be sufficiently different from F_2 to have a lower affinity for the bioassay preparation. However, bioactivity could have been lost in drying and reconstituting the fractions from the Phenyl column, through chemical degradation or by adhesion to the centrifuge tubes.

Several results are consistent with the hypothesis that crayfish POs contain material similar to peptides F_1 and F_2. The POs contain material that co-elutes with F_1 and F_2 on two RP-HPLC systems and that excites the crayfish heart in a manner similar to F_2. Peptide F_1 appears to have cardioexcitatory effects very similar to those of F_2 (Kravitz *et al.* 1987; R. T. Russenes and A. J. Mercier, in preparation). Preliminary results from amino acid analysis of the fractions that co-elute with F_1 and F_2 (I. Orchard and A. J. Mercier, unpublished data) indicate that the amino acids Phe, Leu and Arg are present in the ratios that would be expected for peptides with a carboxy terminal ending of FLRFamide. Thus, the

material is peptidergic and appears to be FMRFamide-like in structure as well as in immunoreactivity. In addition, significant levels of immunoreactivity and bioactivity with longer retention times (33–34 min and 36–44 min) on the Phenyl column suggest the presence of at least two other FaRPs. Further characterization of crayfish FaRPs will require sequence analysis.

The available evidence suggests that FaRPs are involved in modulating cardiac function and in coordinating feeding and digestion. More work is needed to identify the peptides involved and to determine their specific roles.

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