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FMRFamide-LIKE PEPTIDES IN THE LOCUST: DISTRIBUTION, PARTIAL CHARACTERIZATION AND BIOACTIVITY

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Summary

The quantitative distribution of FMRFamide-like peptides in the nervous system and in their putative target sites in the locust *Schistocerca gregaria* is described using radioimmunoassay techniques. The nature of the immunoreactive material has been characterized by high-pressure liquid chromatography. At least six peaks of FMRFamide-like immunoreactivity can be separated in extracts of locust nervous tissue. The relative proportions of these peaks vary from tissue to tissue, suggesting a differential expression of FMRFamide-like peptides in different parts of the locust nervous system. The bioactivity of the endogenous FMRFamide-like peptides has been assessed on the extensor tibiae neuromuscular preparation and on the locust heart. The results suggest that FMRFamide-like peptides in the locust function both as circulating neurohormones and as locally released neuromodulators or neurotransmitters.

Introduction

The neuropeptide Phe-Met-Arg-Phe-NH_2 (FMRFamide) was the first member of a growing interphyletic family of peptides to be isolated and sequenced (see Price *et al.* 1987; Greenberg *et al.* 1988). It was originally isolated from ganglia of the clam *Macrocallista nimbosa* (Price and Greenberg, 1977). More recently a wide range of structurally related *N*-terminally extended neuropeptides has been isolated from a variety of molluscan species (Price *et al.* 1987). Structurally related peptides have also been isolated and sequenced from arthropod nervous systems, such as those of the cockroach *Leucophaea* (Holman *et al.* 1986; Nachman *et al.* 1986*a*,*b*), the lobster *Homarus* (Trimmer *et al.* 1987) and the fruit fly *Drosophila* (Nambu *et al.* 1987). FMRFamide itself is also likely to be present in the nervous system of the leech (Li and Calabrese, 1987). Neuropeptides with a more limited structural similarity to the FMRFamide family have been isolated from coelenterates (Grimmelikhuijzen and Graff, 1986) and even from vertebrate nervous systems (Dockray *et al.* 1983, 1986; Yang *et al.* 1985).

Key words: FMRFamide, neuropeptides, radioimmunoassay, HPLC, bioactivity, insects.

In insects the distribution of FMRFamide-like material has been described immunocytochemically in the nervous system of the locust *Schistocerca* (Myers and Evans, 1985*a*,*b*, 1987) and in that of a variety of other insects (Veenstra, 1984; Veenstra and Schooneveld, 1984; White *et al.* 1986; Brown and Lea, 1988). Furthermore, FMRFamide and related peptides have potent modulatory actions on locust skeletal muscle (Walther *et al.* 1984; Evans and Myers, 1986*a*,*b*) and visceral muscles such as the heart and oviducts (Evans *et al.* 1988, 1989; Cuthbert and Evans, 1989). Recently, the gene coding for the precursor protein from which FMRFamide-like peptides are cleaved has been sequenced in *Drosophila*, indicating that a range of *N*-terminally extended FMRFamide-like peptides, but not FMRFamide itself, may be used as chemical messengers in this species (Schneider and Taghert, 1988). However, it remains to be discovered whether all insect species express the same range of FMRFamide-like peptides. In addition, it is not known if all the FMRFamide immunoreactive cells in the nervous system of a given species express the same range of analogues or if a differential expression occurs in different parts of the nervous system in different species. In addition, the functional roles of each of the endogenous FMRFamide analogues present in the nervous systems of insects and other invertebrates remain to be elucidated.

To begin to answer these questions we have initiated a programme to isolate and identify the FMRFamide-like peptides of the locust nervous system, since in this preparation the cells containing FMRFamide-like immunoreactivity have been localized (Myers and Evans, 1985*a*,*b*, 1987) and several possible roles for these peptides in the modulation of the functioning of skeletal and visceral muscles have been identified (Evans and Myers, 1986*a*,*b*, Evans *et al.* 1988, 1989; Cuthbert and Evans, 1989). In the present paper we report on the quantitative distribution of FMRFamide-like immunoreactivity in different parts of the locust nervous system and in possible target tissues using radioimmunoassay techniques. The separation of this activity into a series of different peak profiles using high-performance liquid chromatography (HPLC) suggests a differential expression of FMRFamide-like peptides in different parts of the nervous system and identified target sites. We also report on the bioactivity associated with several of the immunoreactive peaks.

Materials and methods

Animals

2- to 6-day-old adult male and female locusts *Schistocerca gregaria* were obtained from crowded laboratory cultures fed on wheat seedlings. The animals were then left undisturbed for at least 30 min before use, to minimize any arousal effects.

Tissue extractions

Tissues were dissected rapidly in locust saline (in mmol l^{-1}: NaCl, 150; $CaCl_2$, 4; $KHCO_3$, 4; KH_2PO_4, 6; $MgCl_2$, 2; sucrose, 90, at room temperature), immediately frozen on dry ice and stored at −20°C, or below, prior to extraction.

In preliminary experiments tissues were homogenized in 0.5 ml of either ice-cold acetone or an acidified methanol solution (90 % methanol: 9 % acetic acid: 1 % distilled water). The homogenates were centrifuged for 5 min at 12 000 *g* at room temperature and the pellet resuspended twice in 0.25 ml of extraction solution. The supernatants were pooled and dried in a Speed Vac concentrator (Savant). Dried samples were stored at −20 °C until required and then reconstituted in 100 μl of RIA buffer, or the appropriate solvent for chromatography. The tissue pellets were stored at −20 °C and assayed for their protein content using the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

The acidified methanol extraction technique was the more efficient at extracting FMRFamide-like immunoreactivity from locust tissues (see Results) and was therefore used in all subsequent experiments.

Radioimmunoassay (RIA)

The RIA buffer contained 0.1 mol l^{-1} sodium phosphate (pH 7.4), 0.05 mol l^{-1} NaCl, 0.1 % bovine serum albumin (RIA grade, Sigma) and 0.01 % sodium azide.

Two antisera raised against FMRFamide were used in the course of this work, both of which gave essentially similar results when used to assay HPLC fractions of extracts of locust nervous tissue. The first was obtained from Peninsula (RAS 8755) and each bottle of this rabbit antiserum (enough for 500 RIA tests) was diluted to a final volume of 50 ml in RIA buffer. This serum (characterized by the manufacturer) has similar affinities for FMRFamide and YGGFMRFamide (100 % crossreactivity), low crossreactivity with MRFamide (7 %) and RFamide (<0.01 %) and no crossreactivity with the CCK tetrapeptide. The second antiserum (671M) was used at a final dilution of 1: 10 000 and was kindly supplied by Professor E. Marder. This antiserum has been characterized previously (see Marder *et al.* 1987) and requires the amidated carboxyl terminus for immunoreactivity.

The radioactive tracer used in all experiments was the commercially available ^{125}I-[Tyr^{0}]-FMRFamide (Peninsula Y8773). 10 μCi of this trace (400–1400 Ci mmol^{-1}) was reconstituted in 1 ml of distilled water and further diluted to 100 ml with assay buffer to give approximately 12 000 cts min^{-1} 100 μl^{-1}. Standard amounts of FMRFamide (CRB) and tissue samples were diluted and/or resuspended in a total volume of 100 μl of assay buffer. For the RIA itself, 100 μl of standard/sample was mixed with 100 μl of radiolabelled tracer and 100 μl of diluted antiserum in each assay tube. The tubes were vortexed and incubated at 4 °C for 12–48 h.

Unbound peptide was precipitated by adding 0.2 ml of dextran-coated charcoal solution [0.4 g Norit A activated charcoal (Aldrich) and 0.04 g Dextran (Sigma) in 50 ml of RIA buffer constantly stirred at 4 °C]. The tubes were vortexed and centrifuged at 12 000 *g* for 3 min at room temperature. 400 μl of clear supernatant was counted in an LKB Clini-Gamma counter and the results processed by an attached personal computer. The amounts of FMRFamide-like immunoreactive

material present in the unknown samples were read off a standard curve constructed using authentic FMRFamide as standard.

The inhibition of tracer binding to the antibody was compared for serial dilutions of both FMRFamide standards and locust tissue extracts to ensure a similar crossreactivity with the antiserum.

Gel filtration chromatography

Samples were initially purified by elution with 0.1 mol l^{-1} acetic acid through a G-15 Sephadex (Sigma) column (1.5 cm×45 cm). The void volume of the column was determined by application of Blue Dextran (Sigma) immediately prior to sample application. Eighty 2 ml fractions were collected and 100 μl samples were dried down and assayed by RIA. The column was calibrated with a sample mixture containing 100 pmol each of FMRFamide and YGGFMRFamide.

HPLC analysis

G-15 Sephadex fractions containing FMRFamide-like immunoreactivity were pooled and dried. The samples were resuspended in 2.5 ml of distilled water containing 0.1 % trifluoroacetic acid (TFA), the volume was made up to 6 ml and the samples were applied to a C_{18} reversed-phase Sep-Pak cartridge (Waters) for further purification. The Sep-Pak was activated prior to use with 5 ml of acetone, followed by 5 ml of water and 5 ml of 0.1 % TFA. FMRFamide-like immunoreactive material was eluted from the column in 5 ml of 90 % methanol containing 0.1 % TFA; it was then dried down and resuspended in 100 μl of HPLC solvent.

Three different HPLC solvent systems were used to separate the FMRFamide-like immunoreactive material present in extracts of locust tissues. These were: (1) a 10–40 % acetonitrile gradient plus 0.1 % TFA; (2) a 5–75 % methanol gradient plus 0.1 % TFA; and (3) a 10–40 % acetonitrile gradient plus 0.1 % heptafluorobutyric acid (HFBA). Several gradient profiles were examined for each solvent system to determine the maximum separation of peaks containing FMRFamide-like immunoreactivity. A C_{18} μBondapak (Waters) reversed-phase column was used for all separations. Solvent was run at 1 ml min^{-1} and 1 ml fractions were collected, from which appropriate samples were taken for RIA analysis.

Oxidation of FMRFamide-like immunoreactivity

An extracted tissue sample was split in two. One half was subjected to mild oxidation conditions sufficient to form the sulphoxide derivative of the methionine residue in FMRFamide (Toennies and Callan, 1939). The procedure used was essentially that of Trimmer *et al.* (1987), in which the sample was treated with 0.2 % hydrogen peroxide in the HPLC solvent for 140 min at room temperature prior to direct injection onto the HPLC column. The second half of the sample was made up as above and injected directly onto the HPLC column without any incubation. The samples were eluted from the column using the acetonitrile plus HFBA gradient system and fractions collected for RIA analysis.

Trypsin degradation of FMRFamide-like immunoreactivity

A tissue extract was split in two and treated essentially as described by Trimmer *et al.* (1987). One half was incubated at 37°C for 1 h with 0.05 mg of trypsin (Type 1 from bovine pancreas: Sigma) in sodium phosphate buffer (pH 7.5). The second (control) half of the sample was treated in the same way, but in addition had 0.05 mg of trypsin inhibitor (Type 11–5 from soyabean: Sigma). Tubes were cooled on ice to stop the reaction and trypsin inhibitor added to the experimental samples. 0.05 mg of bovine serum albumin was added to each tube and the samples were then injected onto the HPLC column, eluted using the acetonitrile plus HFBA gradient system, and fractions collected for RIA analysis.

Bioactivity of HPLC fractions containing FMRFamide-like immunoreactivity

HPLC fractions were split into two. One half was assayed for FMRFamide-like immunoreactivity as described above. The other half, after drying down, was resuspended in locust saline and assayed for bioactivity on either the locust extensor tibiae muscle preparation or the locust heart. The effects of the fractions on twitch tension induced by the slow motor neurone were measured in the extensor tibiae muscle, as described previously (Evans and Myers, 1986*b*), to assess FMRFamide-like bioactivity. The effect of fractions on the frequency and amplitude of spontaneous heart contractions was measured in the semi-isolated heart preparation of the locust, as described previously (Cuthbert and Evans, 1989), to assess FMRFamide-like bioactivity.

Results

Radioimmunoassay

Previous immunocytochemical studies have shown FMRFamide-like immunoreactivity to be widely distributed in the locust nervous system (Myers and Evans, 1985*a*,*b*, 1987). The amounts of these peptides were quantified in different parts of the locust nervous system and in potential target sites by use of radioimmunoassay (RIA).

Initially, two different extraction procedures were compared, both of which have been used extensively for the extraction of FMRFamide and FMRFamide-like peptides from various invertebrate tissues. These were extraction into ice-cold acetone (Lehman *et al.* 1984; Price *et al.* 1985; Lehman and Price, 1987) and extraction into acidified methanol (Marder *et al.* 1987; Kobierski *et al.* 1987; Li and Calabrese, 1987). In separate pools of samples of brains and thoracic ganglia, from either male or female locusts, the latter technique consistently extracted more FMRFamide-like immunoreactivity as assayed by RIA. It ranged from 5 to 20 times that extracted by the former technique. Thus, in all other studies reported in this paper, the tissues were extracted into acidified methanol.

Control experiments were performed in which inhibition of tracer binding to the antibody at different serial dilutions was compared for authentic FMRFamide and different tissue extracts. Such dilution curves were almost parallel (Fig. 1),

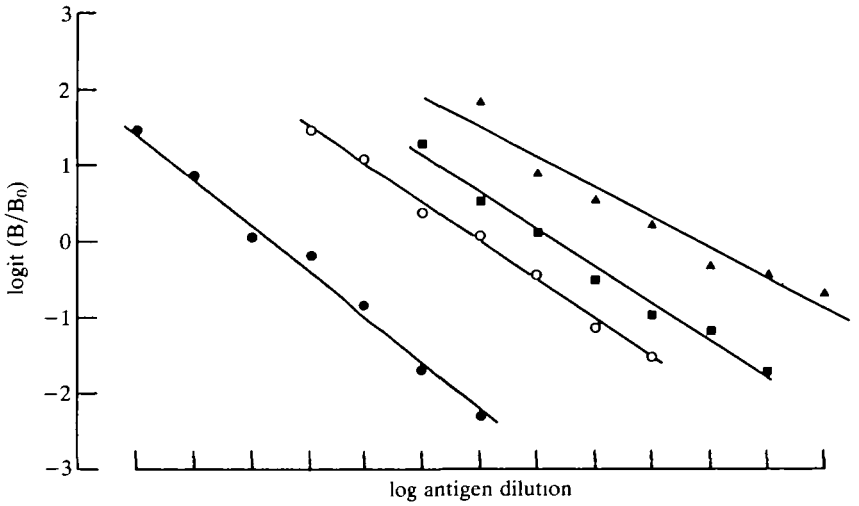

Fig. 1. Inhibition of tracer binding by locust nervous system extracts. Inhibition of tracer binding in the FMRFamide radioimmunoassay using serum 671M by serial dilution of brain (■), thoracic (○) and abdominal (▲) nerve cord extracts is parallel to the inhibition caused by synthetic FMRFamide (●). The ordinate is the logit of the percent tracer bound (B) divided by the maximum amount of tracer bound by the serum (B_0) i.e. logit (B/B_0). The abscissa is the log of the antigen dilution expressed in arbitrary units; a series of twofold dilutions of FMRFamide and the tissue extracts were plotted arbitrarily on a logarithmic scale. FMRFamide was used between 625 and 10 fmol l^{-1} (slope −0.62). Pools of 10 pieces of each tissue were used at the following dilutions: brains, dilution 1:2–1:128 (slope −0.48); thoracic nerve cords, dilution 1:8–1:512 (slope −0.50); abdominal nerve cords, dilution 1:1–1:64 (slope −0.40). Similar curves were obtained for the Peninsula antibody.

indicating that the RIA can be used for the determination of FMRFamide-like immunoreactivity in the various tissue samples as FMRFamide equivalents.

The data shown in Table 1 (obtained using the Peninsula antibody) compare the distribution of FMRFamide-like immunoreactivity in different parts of the nervous system from male and female locusts, and in various potential target tissues. The data are expressed both as the number of FMRFamide-like equivalents per tissue and as its specific activity in terms of the protein content of the tissue. On a per tissue basis the values obtained for samples of female nervous tissue were slightly greater than those from male tissue, but when expressed in terms of the protein content of the tissue this was not apparent, since female locust nervous systems contain more protein than those of males. FMRFamide-like immunoreactivity could be found in all ganglia of the nervous system. Roughly equal amounts, in the range 11–16 pmol mg^{-1} protein, were found in brains and in the prothoracic and mesothoracic ganglia. Slightly less material, 6–9 pmol mg^{-1} protein, was found in the metathoracic ganglion, the terminal abdominal ganglion and the pooled abdominal nerve cords. The metathoracic ganglion contained the same amount of immunoreactive material as the mesothoracic and prothoracic

Table 1. *The distribution of FMRFamide-like immunoreactivity in the nervous system and potential target organs of the locust* Schistocerca gregaria

	FMRFamide equivalents			
	fmol tissue^{-1}		pmol mg^{-1} protein	
	Males (*N*)	Females (*N*)	Males (*N*)	Females (*N*)
Brain	421.5±86.3 (8)	430.9±21.4 (11)	15.2±5.4 (8)	11.3±1.0 (11)
Optic lobe	132.4±32.9 (8)	167.7±10.2 (11)	16.0±5.8 (8)	10.0±2.6 (11)
Suboesophageal ganglion	71.2±14.7 (9)	102.0±4.6 (9)	16.4±4.8 (9)	8.8±0.6 (9)
Prothoracic ganglion	134.0±25.5 (9)	144.0±11.0 (9)	13.4±3.0 (9)	13.3±1.3 (9)
Mesothoracic ganglion	127.9±21.1 (9)	141.3±5.6 (9)	16.2±3.4 (8)	11.7±1.6 (9)
Metathoracic ganglion	117.7±20.7 (9)	141.7±6.7 (9)	7.6±2.3 (9)	9.1±0.9 (9)
Pooled abdominal nerve cords	51.0±9.9 (6)	68.9±3.1 (7)	5.0±1.1 (6)	5.1±0.3 (7)
Terminal abdominal ganglion	39.8±14.0 (5)	33.3±7.2 (5)	6.9±3.2 (4)	5.9±1.4 (5)
Corpora cardiaca	102.9±46.5 (3)	78.6±9.5 (4)	9.0±2.7 (3)	5.6±0.9 (4)
Heart	133.6±24.1 (10)	171.3±9.8 (11)	1.0±0.2 (10)*	1.4±0.1 (11)*
Common oviduct		100.5±19.5 (4)		1.0±0.1 (4)
Lateral oviduct		62.3±5.4 (4)		2.4±1.2 (4)
Loim	6.3±2.3 (3)		0.6±0.3 (3)	
Spm	48.0±7.4 (3)		8.6±2.3 (3)	
Extensor tibiae muscle	33.8±5.6 (8)		0.03±0.01 (8)	
Flexor tibiae muscle	23.2±5.1 (8)		0.05±0.02 (8)	

Values are means±S.E.
Muscle values were obtained from pooled male and female samples.
Loim, lateral oblique intersegmental muscle; Spm, spinopleural muscle.
* Significant difference using Mann–Whitney *U*-test, $P<0.05$.

ganglia on a per tissue basis, but less on a per unit protein basis, probably because the metathoracic ganglion is a fused ganglion containing the first three true abdominal ganglia fused with the true metathoracic ganglion. Significant amounts of immunoreactive material could also be measured in extracts of corpora cardiaca from both male and female locusts. These are important neurosecretory release structures in insects.

Potential target tissues such as the heart and oviducts also contained significant amounts of FMRFamide-like immunoreactive material, indicating they may receive an innervation from neurones containing FMRFamide-like peptides. The increased levels of FMRFamide-like immunoreactivity in female hearts compared

with male hearts was the only statistically significant, sex-related difference we observed. Extracts of the small skeletal muscles, such as the spinopleural muscle and the lateral oblique intersegmental muscle, which receive an innervation by FMRFamide-like immunoreactive neurones (Myers and Evans, 1985*a*), also contained significant amounts of immunoreactive material, detected by RIA. However, the spinopleural muscle samples may have been contaminated by traces of the median neurohaemal organs that run over their surface and which may not have been totally removed during the cleaning process. In contrast, the large leg muscles which receive no FMRFamide-like innervation, such as the extensor and flexor muscles of the tibia, contained very little, if any, immunoreactive material, determined by RIA, when the results were expressed on a unit protein basis. Indeed, in individual assays, values for these tissues were always at the threshold of determination.

Samples of haemolymph from both male and female locusts consistently showed the presence of FMRFamide-like material, indicating its possible role as a circulating neurohormone in the locust. Estimates made on 1-ml samples of haemolymph showed that females contained $8.3\pm2.7\,\text{nmol}\,l^{-1}$ of FMRFamide equivalents (mean±S.E., $N=6$), whilst males contained $5.3\pm1.4\,\text{nmol}\,l^{-1}$ of FMRFamide equivalents (mean±S.E., $N=5$). The values we obtained for haemolymph samples showed a large variation, from 1.7 to $20.4\,\text{nmol}\,l^{-1}$, suggesting that different amounts of FMRFamide-like material may be released into the haemolymph of locusts under different conditions and at different ages. A detailed study of this is currently being carried out.

HPLC analysis of immunoreactivity

The radioimmunoassay results described above indicate a differential distribution of FMRFamide-like immunoreactivity in different parts of the locust nervous system and in potential target organs. To examine the composition of this immunoreactive material, tissue extracts were subjected to gel chromatography followed by reversed-phase HPLC analysis.

Initially, acidified methanol extracts were made of pools of locust brains and these extracts were chromatographed on a G-15 Sephadex column, with the fractions assayed by RIA. The elution profiles of these columns with $0.1\,\text{mol}\,l^{-1}$ acetic acid (Fig. 2A) show that the bulk of the FMRFamide-like immunoreactivity, accounting for more than 80 % of the total activity put on the column, eluted in a broad peak between fractions 24 and 39. This is considerably earlier than FMRFamide standards, which eluted between fractions 52 and 70 when run on the same column (data not shown). This suggests that the bulk (perhaps all) of the FMRFamide-like immunoreactivity detected in locust nervous tissue by RIA, and by immunocytochemistry, is due to the presence of peptides with a higher relative molecular mass than FMRFamide itself. The small immunoreactive peak eluting just after the void volume disappeared on further HPLC analysis and was not studied further. In control experiments, up to 160 1-min fractions were collected to see if the locust brain extracts contained any immunoreactive material eluting a

5–6 void volumes, similar to the so-called 'pseudoFMRFamide' found in *Helix* tissue extracts (Lehman and Price, 1987). No such activity was found in the locust brain extracts or in extracts of any of the other locust tissues examined. More than 90% of the immunoreactivity applied to the Sephadex columns was recovered in the fractions analysed.

The major fractions (24–39) of the broad main immunoreactive peak were pooled and partially purified on C_{18} Sep-Pak cartridges and then further analysed by reversed-phase HPLC. Initially, HPLC columns loaded with brain extracts were eluted with a 10–30% acetonitrile gradient containing 0.1% TFA. This revealed the presence of six consistent peaks of immunoreactivity, none of which co-chromatographed with authentic FMRFamide or YGGFMRFamide (Fig. 2B). We have named these peaks *A–E* based on their respective elution times, but it should be emphasized that we have done this for convenience of comparison between different solvent systems and different tissue samples. It does not imply that each peak contains a single FMRFamide-like peptide since, at best, this approach can only give a minimum estimate of the number of FMRFamide-like peptides present in an extract.

In the locust brain extract (Fig. 2B) the two major peaks of immunoreactivity, *C* and *E*, were well separated from each other by the 10–30% acetonitrile plus 0.1% TFA solvent system. However, peak *C* was not well resolved from the two initial peaks *A* and *B* which were consistently present. A fifth immunoreactive peak, *D*, eluted between *C* and *E*, and frequently appeared to consist of two closely eluting peaks which were not completely resolved. The sixth immunoreactive peak, *F*, was present in most brain extracts but varied considerably in size in extracts of different batches of animals.

Several other solvent systems and gradient profiles were employed to maximize the separation of the six immunoreactive peaks identified above and to see if any further peaks could be resolved. Fig. 2C shows that a 5–60% methanol plus 0.1% TFA gradient, which has been used by previous workers to separate FMRFamide-like peptides on reversed-phase HPLC (e.g. Li and Calabrese, 1987), did not improve the resolution of the peaks. Similarly, a 5 mmol l^{-1} sodium phosphate pH 7.0 buffer with a 25–45% acetonitrile gradient (Price *et al.* 1985) did not improve the separation. However, we obtained a substantial improvement in the separation of the six immunoreactive peaks by use of a 10–40% acetonitrile plus 0.1% HFBA gradient (Fig. 2D), which has been used successfully in the separation of small cardioactive peptides (SCPs) from molluscan tissue (Lloyd *et al.* 1987). This gradient clearly separated peaks *A* and *B* from peak *C* and gave a good separation of the other peaks. However, it did not allow us consistently to separate the two components of the peak we designated *D*. We established the relationship between the different immunoreactive peaks in the different solvent systems by isolating the separate immunoreactive peaks from a brain extract run on an acetonitrile plus 0.1% HFBA gradient and running them separately in the other solvent systems. On the basis of the above results we decided to use the acetonitrile plus 0.1% HFBA gradient system to analyse the FMRFamide-like

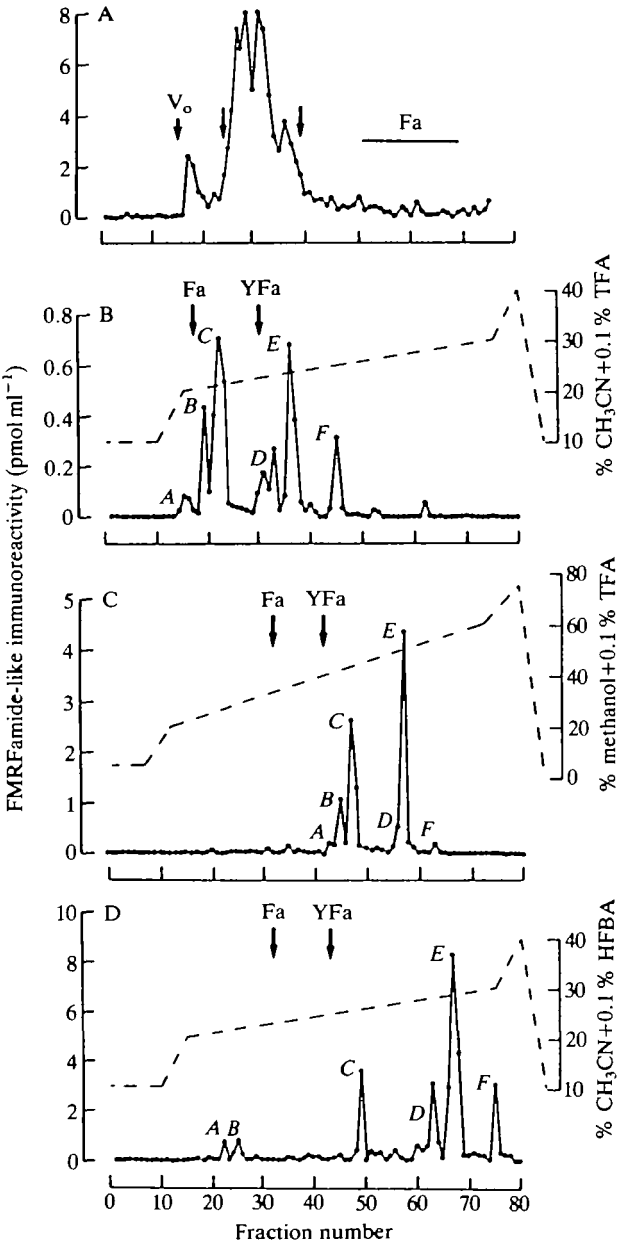

Fig. 2

immunoreactivity in the different parts of the locust nervous system and possible target tissues.

To analyse the chemical nature of the immunoreactive peaks present in the brain extracts, we performed a number of control experiments (Fig. 3). The pattern of immunoreactivity from half a brain extract run on an acetonitrile plus 0.1% HFBA gradient is shown in Fig. 3A, whilst Fig. 3B shows the profile for the other half of the extract which was subjected to mild oxidation conditions (se

Fig. 2. Chromatographic characterization of FMRFamide-like immunoreactivity from extracts of locust brains. (A) Application of extract to a G-15 Sephadex column (45 cm long and 1.5 cm in diameter). V_o indicates the elution position of Blue Dextran (void volume). Fractions between the longer arrows were pooled and dried down for HPLC analysis. Brain extracts were run in three different HPLC gradient systems, a 10–40 % CH_3CN plus 0.1 % TFA gradient (B), a 5–75 % methanol plus 0.1 % TFA gradient (C) and a 10–40 % CH_3CN plus 0.1 % HFBA gradient (D). The six peaks designated *A–F* represent the six major immunoreactive peaks consistently found in locust nervous tissue extracts. Fa and YFa indicate the elution positions of FMRFamide and YGGFMRFamide, respectively, which do not co-elute with any of the peaks containing FMRFamide-like immunoreactivity in the extracts. The samples run in A,B,C,and D correspond to 200, 17, 50 and 50 brain equivalents, respectively.

Materials and methods). Peaks *B*, *C* and *D* were almost completely destroyed by this treatment, whilst peak *E* was reduced by 50 %. In contrast, the amount of material co-eluting with peak *A* and in an unidentified peak following peak *C* (Fig. 3B) was substantially increased, suggesting these peaks might represent oxidation products of the material in the other peaks. The susceptibility of the main peaks to this form of oxidation indicates that the major peptides in them are likely to contain an amino acid, such as methionine, which can easily be oxidized under the conditions used (Toennies and Callan, 1939; Croft, 1974; Trimmer *et al.* 1987). The lower susceptibility of peak *E* to this form of oxidation could be explained if it contains a mixture of peptides, only some of which are susceptible to oxidation. Essentially similar results were obtained by the oxidation of extracts from thoracic nerve cords, except that peak *C* was only reduced by 50 %. This could again be explained if this peak contains a mixture of peptides only some of which are susceptible to the mild oxidation.

To confirm the peptidergic nature of the immunoreactive peaks *A–F*, isolated from the locust brain by reversed-phase HPLC, we subjected them to proteolytic enzymatic digestion using trypsin (see Materials and methods). Fig. 3C shows the immunoreactive profile of half a brain extract run on an acetonitrile plus 0.1 % HFBA gradient after being subjected to enzymatic digestion by trypsin in the presence of soyabean trypsin inhibitor. It is not substantially different (except for an increased relative size of peak *B* in this sample) from the controls (see Figs 2D and 3A). However, in the absence of the soyabean trypsin inhibitor, the other half of the extract (Fig. 3D) shows an almost total destruction of all the FMRFamide-like immunoreactivity in all the peaks *A–F*, demonstrating their peptidergic nature.

Distribution of immunoreactive peaks in different tissues

The presence of at least six consistent peptidergic immunoreactive peaks on HPLC analysis of extracts of locust brains raises the question of how they relate to the differential quantitative values obtained for extracts of different tissues using radioimmunoassay. The differences could be due to a differential expression of the same immunoreactive pattern in extracts of all tissues or to a differential

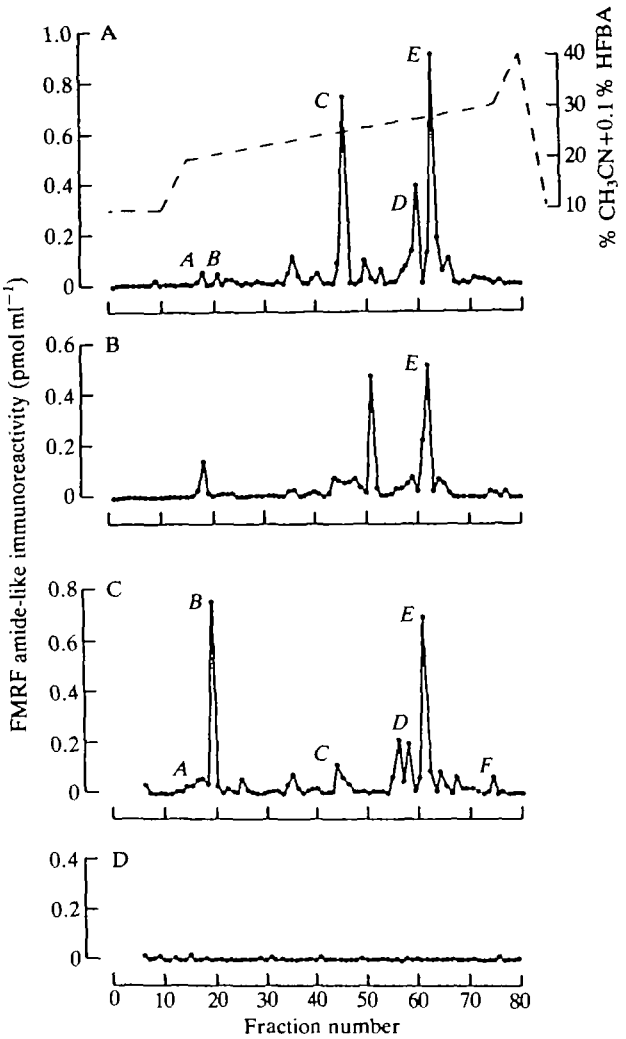

Fig. 3. Properties of FMRFamide-like immunoreactive peaks from locust brain extracts (each corresponding to 25 brain equivalents) subjected to HPLC on a 10–40% CH_3CN plus 0.1% HFBA gradient. (A) Control half of sample for oxidation experiment. (B) Other half of extract shown in A which was subjected to mild oxidation, substantially reducing, but not completely eliminating, many of the major peaks. (C) Control half of sample for proteolytic digestion experiment; the extract was incubated with trypsin in the presence of soyabean trypsin inhibitor. (D) Experimental half of same sample as in C incubated with trypsin alone, showing complete removal of immunoreactive peaks.

expression of the different peaks in different tissues and target sites. We have examined this question in detail using reversed-phase HPLC analysis and the acetonitrile plus 0.1% HFBA gradient, since such information may suggest functional roles for each of the immunoreactive peaks.

A comparison of the immunoreactive profile of tissue extracts from pools of

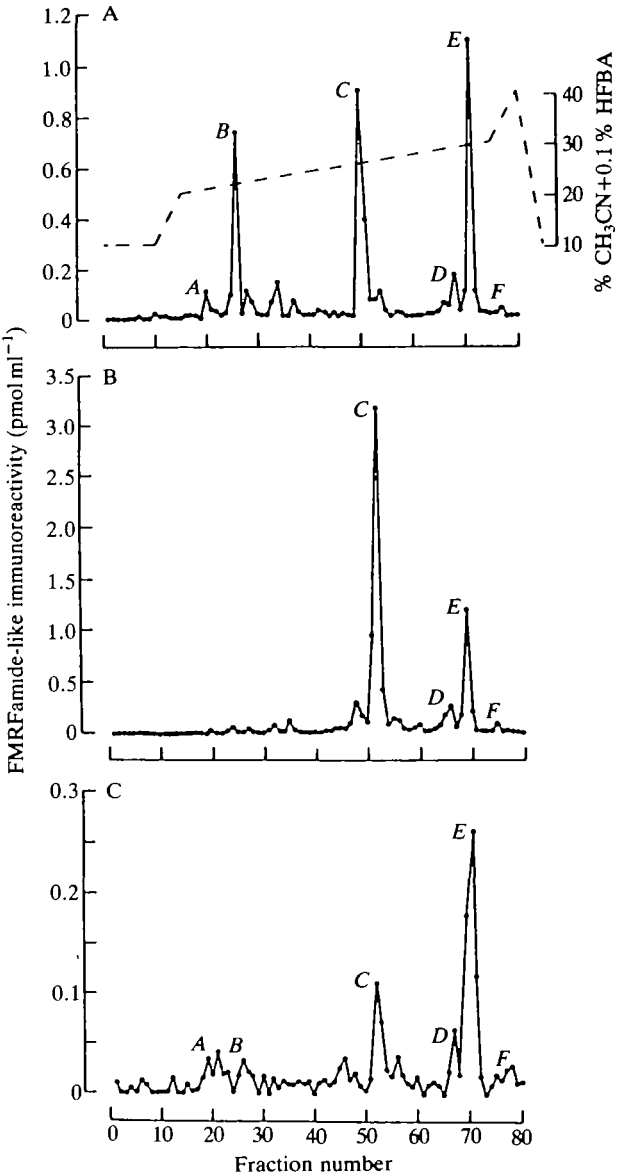

Fig. 4. A comparison of the FMRFamide-like immunoreactive profiles of extracts (each corresponding to 50 tissue equivalents) from different parts of the locust nervous system subjected to HPLC analysis on a 10–40 % CH_3CN plus 0.1 % HFBA gradient, indicating a differential expression of immunoreactive peaks. (A) Brain extract; (B) thoracic nervous system extract; and (C) abdominal nerve cord extract.

locust brains, thoracic nerve cords and abdominal nerve cords (Fig. 4A–C) revealed a differential expression of the immunoreactive peaks in different parts of the locust nervous system. In extracts of thoracic nerve cords (Fig. 4B) peak *C* was

the major peak present, with smaller amounts of peaks *E* and *D*. In contrast, in both the brain and abdominal nerve cords there was relatively much more peak *E* compared with peak *C* than in the thoracic nerve cords. In addition, in the thoracic nerve cords there was relatively less of peaks *A* and *B*. The immunoreactive profile obtained from the abdominal nerve cords (Fig. 4C) was similar to that found in brain extracts, but quantitatively much smaller amounts of each peak were present, confirming the data obtained from the quantitative radioimmunoassay studies (Table 1). No qualitative differences were found in the immunoreactive patterns obtained from pools of male and female brains, thoracic nerve cords or abdominal nerve cords (data not shown).

Our previous immunocytochemical studies have indicated an association of FMRFamide-like material with the major neurohaemal organs of the locust, namely the corpora cardiaca and the median neurohaemal organs of the thoracic ganglia (Myers and Evans, 1985*a*,*b*, 1987). The HPLC immunoreactive profile of extracts from pools of male and pools of female corpora cardiaca were compared with those of a brain extract run immediately after them (Fig. 5). In the case of both the male and female corpora cardiaca extracts only peaks *D* and *E* were detectable, with 2–3 times as much peak *E* as peak *D*. No sex-related differences were apparent.

In an attempt to assign functions to some of the immunoreactive peaks observed in tissue extracts of nervous tissue, we compared the immunoreactive profiles obtained from a variety of potential target sites for the actions of FMRFamide-like peptides in the locust, including the heart (Evans *et al.* 1988; Cuthbert and Evans, 1989), oviducts (Evans *et al.* 1989) and various skeletal muscles (Myers and Evans, 1985*a*; Evans and Myers, 1986*a*,*b*).

The HPLC immunoreactive profiles from pools of male and female hearts showed sex-related differences (Fig. 6A and B). In addition, the immunoreactive peaks were bigger in the female extracts than in the male ones, confirming the differences observed in the quantitative radioimmunoassay studies (Table 1). In the heart extracts the pattern of the immunoreactive peaks *A*–*E* (the peaks previously extracted from nervous tissue) was similar in both males and females, except for a much increased level of peak *B* in the female extracts. However, in addition to these peaks, three new peaks (labelled *H*1, *H*2 and *H*3 on the basis of their elution times) could be consistently identified which were not present in nervous tissue extracts. One of these, *H*3, was present in much larger amounts in extracts of female hearts compared with those of males. Extracts from locust oviducts also contained immunoreactive material that co-eluted with peak *H*3 from the heart samples and with peaks *D* and *E*, which were found in both the heart and nervous tissue extracts.

FMRFamide-like immunoreactivity has also been identified in neurones which project from the median nerve to run over the surfaces of several small skeletal muscles that degenerate in the locust after the first few days of adult life (Myers and Evans, 1985*a*). The immunoreactive HPLC profiles of pools of two of these small muscles, the spinopleural muscle and the lateral oblique intersegmental

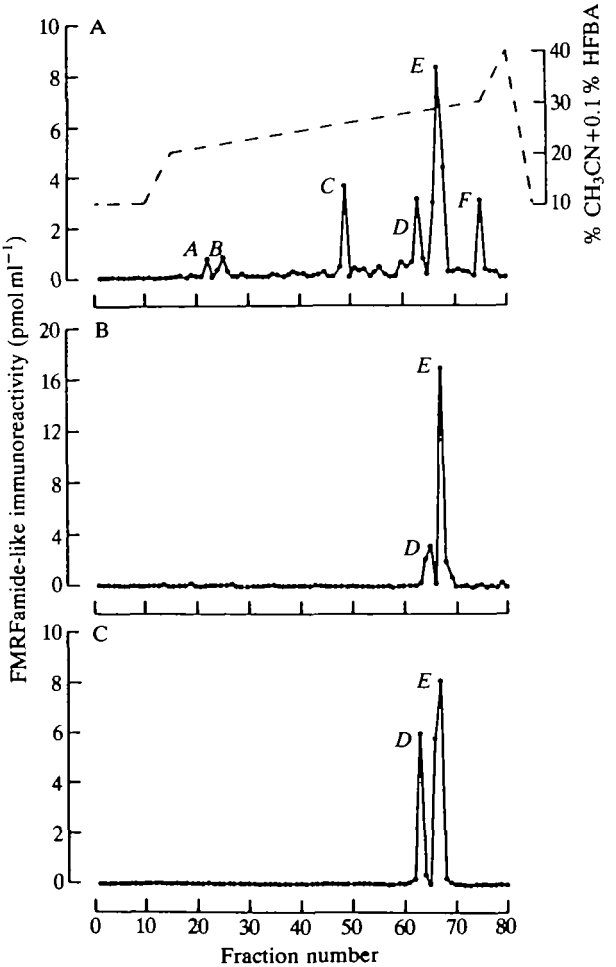

Fig. 5. A comparison of the FMRFamide-like immunoreactive profiles of extracts (each corresponding to 50 tissue equivalents) from locust brain (A), female corpora cardiaca (B) and male corpora cardiaca (C) subjected to HPLC analysis on a 10–40 % CH_3CN plus 0.1 % HFBA gradient. Two of the major immunoreactive peaks in the brain are also present in the corpora cardiaca, which are important neurohaemal release sites for brain neurosecretory materials.

muscle, from newly moulted adult locusts, were compared with that of a sample of thoracic nerve cords run immediately before them (Fig. 7). In the extract of the spinopleural muscle (Fig. 7B) immunoreactive material co-eluted with peak *C* from the thoracic sample and there was a relative enrichment of the two peaks eluting prior to peak *C* in the thoracic sample. In addition, there was much less immunoreactive material co-eluting with peaks *D* and *E* than in the thoracic sample. However, as pointed out above, because the neurohaemal organ of the locust median nerve runs over the surface of this muscle it is impossible to rule out the possibility that some of the immunoreactive material obtained from extracts of

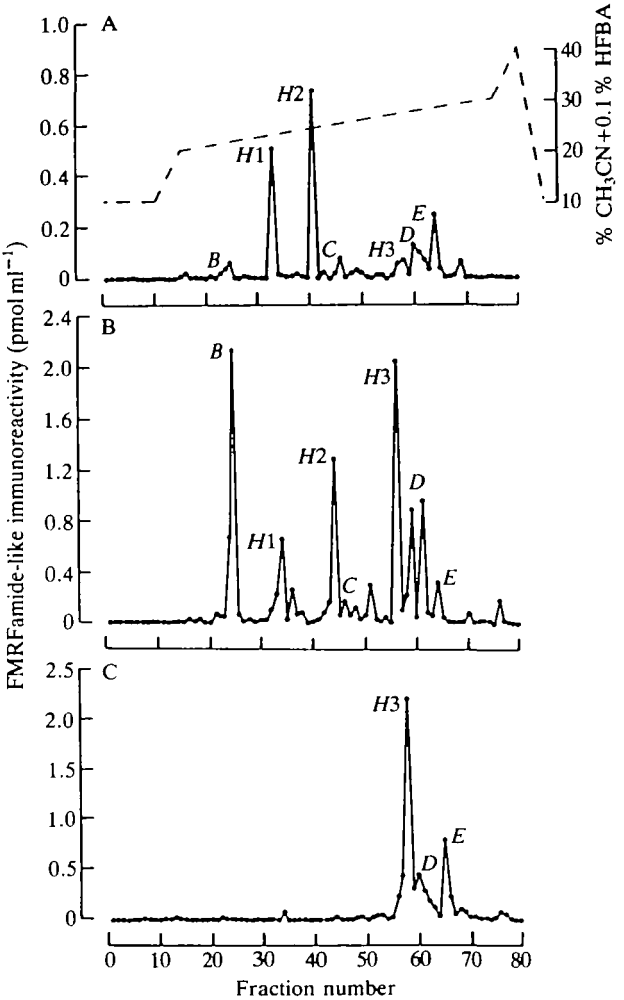

Fig. 6. A comparison of the FMRFamide-like immunoreactive profiles of locust extracts (each corresponding to 50 tissue equivalents) from male hearts (A), female hearts (B) and oviducts (C) subjected to HPLC analysis on a 10–40 % CH_3CN plus 0.1 % HFBA gradient. Some of the immunoreactive peaks in these tissues containing visceral muscle co-migrate with peaks from nervous tissue extracts, but others (*H1*, *H2* and *H3*) are novel.

this muscle may be the result of contamination from small fragments of neurohaemal organ which were not removed during the cleaning process. In contrast, the lateral oblique intersegmental muscle lies at a considerable distance from the median neurohaemal organs and is thus not at risk from such contamination. Thus, it is more likely to exhibit a pattern of FMRFamide-like immunoreactivity related to its function as a neuromodulator or neurotransmitter in this muscle. Fig. 7C indicates that the HPLC immunoreactive profile of this muscle contains material that co-elutes with peaks *C*, *D* and *E* of the thoraci

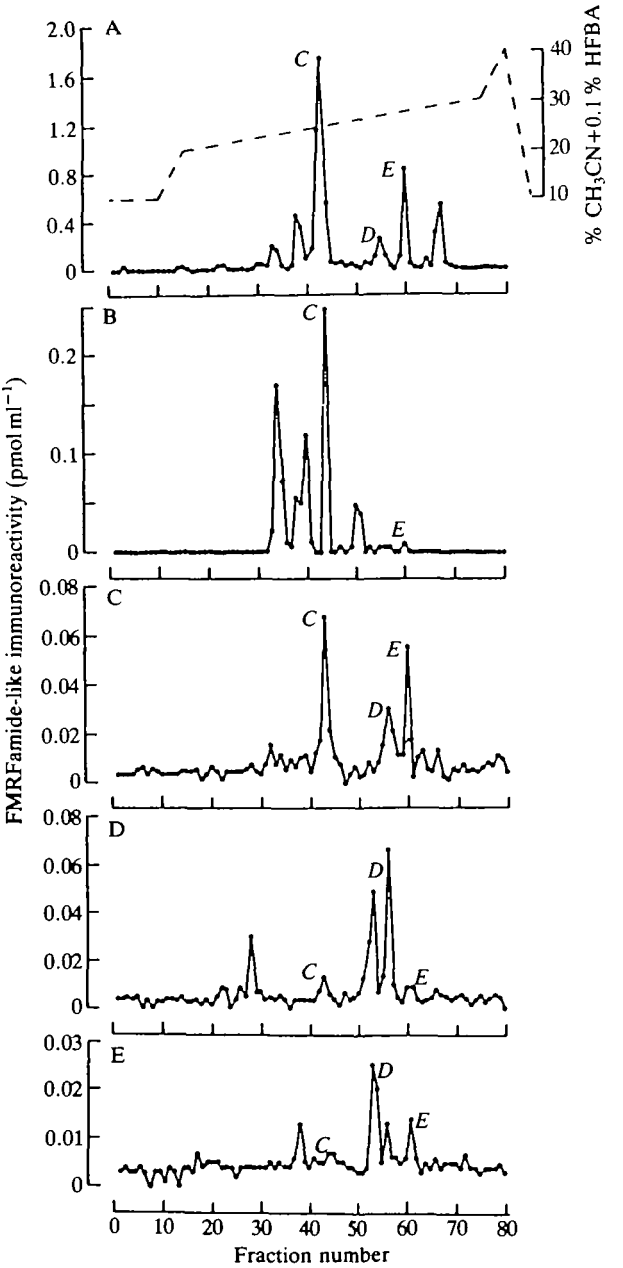

Fig. 7. A comparison of the FMRFamide-like immunoreactive profiles of locust extracts from thoracic nerve cords (A), spinopleural muscles (B), lateral oblique intersegmental muscles (C), female haemolymph (D) and male haemolymph (E) subjected to HPLC analysis on a 10–40 % CH_3CN plus 0.1 % HFBA gradient. Some of the peaks in the skeletal muscle and haemolymph extracts co-migrate with the peaks of the thoracic nerve cord extracts. The samples run in A, B and C correspond to 25, 100 and 100 tissue equivalents, respectively, and those in D and E correspond to 1 ml of haemolymph.

nerve cord extracts, with a relative enrichment of the material co-eluting with peak *D*.

If any of the peptides in the FMRFamide-like immunoreactive peaks function as circulating neurohormones in the locust, released from either the corpora cardiaca or the median neurohaemal organs, as has been suggested by Myers and Evans (1985*a*,*b*, 1987), it should be possible to detect such peptides in the haemolymph. Fig. 7D,E indicates that this is indeed the case and that more immunoreactive material was obtained from the same volume (1 ml) of haemolymph from females than from males, using mature adult animals. In both cases the pattern of immunoreactive peaks was similar, the major peak co-eluting with the *D* peak identified in nervous tissue extracts. In both male and female haemolymph samples the *D* peak was clearly differentiated into two closely eluting components, as was sometimes seen in samples from nervous tissue. Further investigation is needed to establish the relationship between the two closely related *D* peaks and the *H*3 peak identified in heart and oviduct samples, which has not been isolated from nervous tissue extracts. The haemolymph samples also contained material co-eluting with peaks *C* and *E* from nervous tissue samples and each had a different unknown peak eluting between the positions of the *B* and *C* peaks from nervous tissue.

Bioactivity of immunoreactive peaks

HPLC separation of immunoreactive peaks cannot alone distinguish between peaks containing functionally active peptides, their larger inactive precursors or their inactive metabolic breakdown products. To identify the peaks of FMRF-amide-like immunoreactivity isolated from the locust nervous system which contain physiologically active peptides it is essential to determine which peaks exhibit bioactivity on potential target tissues. Thus, we have surveyed the bioactivity present in HPLC-separated fractions by application to two locust preparations known to be sensitive to FMRFamide-related peptides, the extensor tibiae muscle of the locust hindleg (Walther *et al.* 1984; Evans and Myers, 1986*a*,*b*; Cuthbert and Evans, 1989) and the locust heart (Cuthbert and Evans, 1989). This approach does not preclude the possibility that immunoreactive peaks not found to be active on these preparations may be physiologically active on locust preparations not tested.

The extensor tibiae muscle bioassay revealed FMRFamide-like bioactivity capable of increasing the amplitude and relaxation rate of twitch tension evoked by the slow motor neurone co-migrating with immunoreactive peaks *C*, *D* and *E* of a thoracic nerve cord extract run in the acetonitrile plus 0.1% HFBA gradient system (Fig. 8). Additional bioactivity was obtained in the small peak eluting prior to peak *C* and also in the region of the chromatogram prior to peak *D*. Further, bioactivity was also detected much earlier in the chromatogram, associated with fraction 21, which did not contain any FMRFamide-like immunoreactivity. This raises the possibility that other peptides not structurally related to FMRFamide may exhibit FMRFamide-like bioactivity in the extensor tibiae muscle preparation

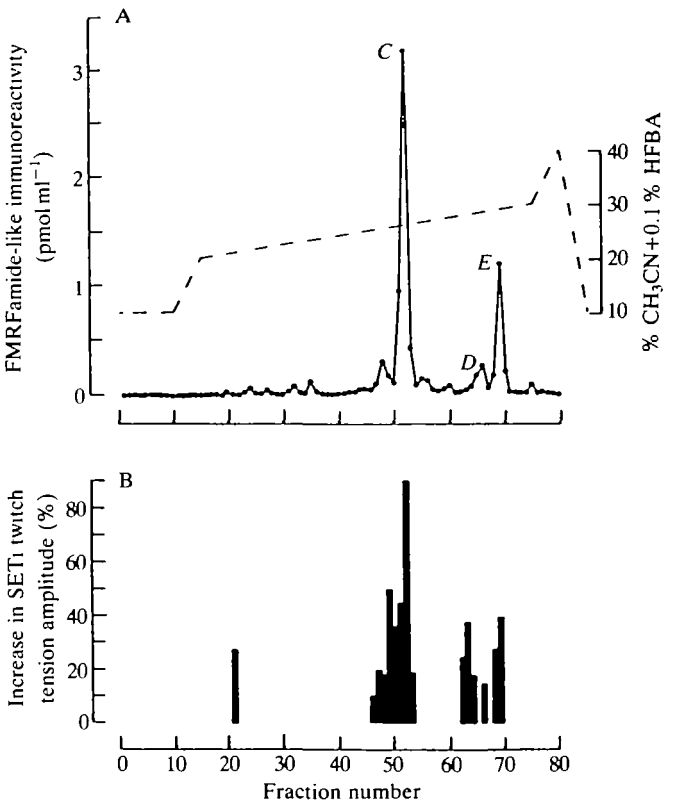

Fig. 8. A comparison of the FMRFamide-like immunoreactive profile and the bioactivity on the locust hindleg extensor tibiae muscle preparation of HPLC fractions obtained from a thoracic nerve cord extract (50 tissue equivalents) run on a 10–40 % CH_3CN plus 0.1 % HFBA gradient.

and emphasizes the point that FMRFamide-like bioactivity on the extensor tibiae muscle cannot alone be used to identify unequivocally peaks containing FMRFamide-like peptides. This raises doubts about the interpretation of the results from studies, such as that of Schiebe *et al.* (1988), where the latter approach was the only one used.

The bioactivity associated with peaks *C*, *D* and *E* in the thoracic nerve cord extracts run in the acetonitrile plus 0.1 % HFBA gradient system also co-chromatographed with the same peaks in the acetonitrile plus 0.1 % TFA and methanol plus 0.1 % TFA gradient systems. In addition, the same bioactivity was found to be associated with these same peaks in extracts of locust brains and abdominal ganglia.

The locust heart bioassay also revealed FMRFamide-like bioactivity co-migrating with several of the immunoreactive peaks of a thoracic nerve cord extract run in the acetonitrile plus 0.1 % HFBA gradient system (Fig. 9). Peak *C* produced cardioinhibition in the locust heart, both in terms of the amplitude of the

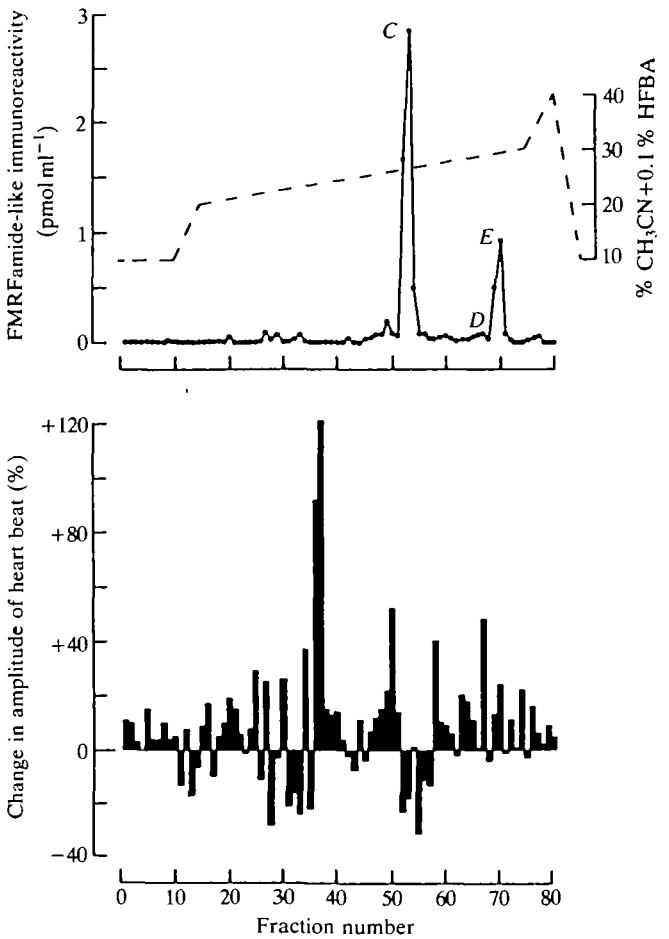

Fig. 9. A comparison of the FMRFamide-like immunoreactive profile and the bioactivity on the locust semi-isolated heart preparation of HPLC fractions obtained from a thoracic nerve cord extract (50 tissue equivalents) run on a 10–40% CH_3CN plus 0.1% HFBA gradient.

heart beat and in terms of the frequency (not shown) of its spontaneous contractions. In contrast, peak *D*, and to a lesser extent peak *E*, produced cardioexcitation, both in terms of the amplitude of the heart beat and in terms of the frequency (not shown) of its spontaneous contractions. Further, the locust heart bioactivity associated with peaks *C*, *D* and *E* also co-migrated with the same immunoreactive peaks when chromatographed on an acetonitrile plus 0.1% TFA gradient system (not shown). The thoracic nerve cord extract also showed cardioexcitatory and cardioinhibitory bioactivity that did not co-migrate with any of the peaks showing FMRFamide-like immunoreactivity in both of the above solvent systems. This suggests, not surprisingly, that peptides other than those

with structural similarities to FMRFamide may also be involved in cardioregulation in the locust.

Discussion

The present paper reports on the isolation, partial characterization and bioactivity of FMRFamide-like peptides in the nervous system of the locust. It complements previous studies on this family of peptides in this preparation, in which cells containing FMRFamide-like peptides were identified immunocytochemically (Myers and Evans, 1985*a*,*b*, 1987) and in which a pharmacological characterization was carried out for receptor sites for such peptides on various potential target sites, such as skeletal and visceral muscle (Walther *et al.* 1984; Evans and Myers, 1986*a*,*b*; Evans *et al.* 1988, 1989; Cuthbert and Evans, 1989). Our radioimmunoassay studies indicate that FMRFamide-like peptides are widespread in the locust nervous system and in various potential target sites, such as heart, oviducts and various skeletal muscles. They also indicate that their distribution is not uniform, with higher concentrations being found in the brain and thoracic regions, and lower amounts in the abdominal regions of the nervous system. In addition, specific skeletal muscles, such as the spinopleural and lateral oblique intersegmental muscles, which have previously been shown to receive an FMRFamide-like immunoreactive innervation (Myers and Evans, 1985*a*), have much higher levels of these peptides than larger muscles, such as the extensor tibiae and flexor tibiae muscles of the hindleg, which do not receive such an innervation. HPLC fractionation of locust tissue extracts reveals at least six immunoreactive peaks, the relative amounts of which vary considerably from one tissue to another and in different regions of the locust nervous system. We have recently isolated and sequenced the major FMRFamide-like peptide present in extracts of thoracic nerve cords of sexually mature adult locusts which corresponds to peak *E* described in the present paper. The primary sequence of this peptide is Pro-Asp-Val-Asp-His-Val-Phe-Leu-Arg-Phe-NH_2 and the bioactivity of the native and synthetic neuropeptide and that of peak *E* are identical on both the locust heart and the hindleg extensor tibiae muscle bioassays (Robb *et al.* 1989). The correlation of bioactivity in the locust extensor tibiae muscle and heart bioassays with the three major immunoreactive peaks suggests possible physiological roles for some of the peptides contained in these peaks. Further, the finding that additional peaks of FMRFamide-like bioactivity occur in HPLC fractions of tissue extracts, tested in both bioassays, which are not correlated with FMRFamide-like immunoreactivity, suggests that other peptides not structurally related to FMRFamide can also produce similar actions on these bioassay systems. It also suggests that studies (e.g. Schiebe *et al.* 1988) in which FMRFamide-like bioactivity has been used as the sole criterion for the identification of HPLC peaks containing FMRFamide-like peptides should be interpreted with caution.

The results of the present study agree well with our previous immunocytochemical studies (Myers and Evans, 1985*a*,*b*, 1987). Both approaches indicate high levels

of immunoreactive material in the brain and thoracic nerve cords and lower amounts in the abdominal ganglia. The amounts of FMRFamide-like material present in locust nervous tissue are in the same range as those found in lobster nervous tissue when expressed on a per tissue basis for ganglia of the ventral nerve cord, but are an order of magnitude higher when expressed on a per milligram protein basis, probably because of the smaller size of the insect ganglia (cf. Kobierski *et al.* 1987). As in lobster (Trimmer *et al.* 1987) and crab (Marder *et al.* 1987) ganglia, HPLC analysis of locust nervous tissue extracts indicates that multiple peaks of FMRFamide-like immunoreactivity are present, none of which co-migrate with authentic FMRFamide or YGGFMRFamide. In the locust, the major peaks of FMRFamide-like immunoreactivity are likely to consist of *N*-terminally extended peptides, some of which probably contain a methionine residue because of their susceptibility to mild oxidation conditions (Toennies and Callan, 1939; Croft, 1974). This contrasts with the lobster studies where the major *N*-terminally extended peptides were not susceptible to mild oxidation and which, on sequencing, were tentatively identified as *N*-terminally extended FLRFamide analogues (Trimmer *et al.* 1987). The results of both the present radioimmunoassay study, and our previous immunocytochemical studies (Myers and Evans, 1985*a*,*b*, 1987), indicate that FMRFamide-like peptides in the locust are likely to function both as circulating neurohormones and as neurotransmitters or locally released neuromodulators.

A neurohormonal role for FMRFamide-like peptides in the locust is suggested because of their immunocytochemical localization in well-known neurosecretory release structures such as the corpora cardiaca and the median neurohaemal organs of the thoracic nervous system (Myers and Evans, 1985*a*,*b*, 1987). The present paper has shown that large quantities of FMRFamide-like material are present in the corpora cardiaca of both male and female locusts and that, upon HPLC analysis, this can be seen to correspond to two specific peaks (*D* and *E*) of the immunoreactive material found in locust brains, suggesting that the material contained in these peaks may be released into the circulation as neurohormones. In addition, the relatively increased levels of peak *C* material in the thoracic regions of the nerve cord, combined with the immunocytochemical localization of FMRFamide-like material in the median neurohaemal organs, suggests that peak *C* material might be released into the haemolymph from these organs. The above conclusions are supported by the finding of FMRFamide-like immunoreactivity in locust haemolymph samples which co-migrate with peaks *C*, *D* and *E*. The range of haemolymph concentrations (1.7–20.4 nmol l^{-1}) found for circulating levels of FMRFamide-like peptides in the locust is similar to that found for FMRFamide-like material in the blood of the lobster (0.01–0.1 nmol l^{-1})(Kobierski *et al.* 1987), the snail *Helix* (2.6–56.8 nmol l^{-1}) (Price *et al.* 1985) and the clam *Macrocallistica* (0.8–1.4 nmol l^{-1}) (Nagle, 1982). In the lobster, FMRFamide-like immunoreactivity has been shown to be associated with the pericardial organs, well-known crustacean neurosecretory release structures (Kobierski *et al.* 1987).

Circulating levels of FMRFamide-like peptides in the locust may also functio

as cardioregulatory peptides. Extracts of locust nervous tissue have cardioinhibitory activity associated with immunoreactive peak *C* and cardioexcitatory activity associated with peaks *D* and *E* of nervous tissue extracts run on HPLC. In addition, the threshold concentrations of the most active FMRFamide analogues for producing cardioexcitation (lobster peptide F1, Trimmer *et al.* 1987) and cardioinhibition (cockroach peptide leucomyosuppressin, Holman *et al.* 1986) occur in the range 10^{-9}–10^{-10} mol l^{-1} (Cuthbert and Evans, 1989), which is well within the physiological levels measured in the present study. FMRFamide-like peptides were originally described as cardioexcitatory regulators of molluscan hearts (Price and Greenberg, 1977) and have since been shown to be capable of modulating the activity of leech hearts (Kuhlman *et al.* 1985*a*,*b*) and the activity of hearts from arthropods such as *Limulus* (Watson *et al.* 1984), the blue crab *Callinectes* (Krajniak and Greenberg, 1988) and the lobster *Homarus* (E. A. Kravitz, B. A. Trimmer and M. F. Goy, unpublished observations quoted in Kobierski *et al.* 1987).

FMRFamide-like peptides released into the circulation, in addition to their modulating effects on visceral muscle, are also likely to modulate certain skeletal muscles which do not appear to receive a direct FMRFamide-like neuronal innervation and which have been shown to be extremely sensitive to the application of FMRFamide analogues, especially the *N*-terminally extended ones (Evans and Myers, 1986*a*,*b*; Cuthbert and Evans, 1989). In the extensor tibiae muscle of the locust hindleg, FMRFamide and related analogues increase the amplitude of slow motor neurone-induced twitch tension, as well as the rates of contraction and relaxation. The most potent analogue tested was TNRNFLRFamide, one of the naturally occurring FMRFamide-like peptides extracted from the lobster (Trimmer *et al.* 1987), which has a threshold for action on the extensor muscle of between 10^{-11} and 10^{-12} mol l^{-1} (Cuthbert and Evans, 1989). This is again very much lower than the estimated circulating levels of FMRFamide-like peptides in locust haemolymph, suggesting that this class of circulating neuropeptides may exert a variable tonic modulation of the properties of locust skeletal muscles. FMRFamide and related peptides also produce a range of effects on molluscan somatic muscles, such as the pharyngeal and tentacle retractor muscles of *Helix* (Cottrell *et al.* 1983*a*; Lehman and Price, 1987) and certain buccal mass muscles of *Aplysia* (Richmond *et al.* 1986). Exoskeletal muscles of the lobster also respond to one of the forms of endogenous FMRFamide-like peptides in concentrations within an order of magnitude of those measured in haemolymph (E. A. Kravitz, B. A. Trimmer and M. F. Goy, unpublished observations, quoted in Kobierski *et al.* 1987).

In the locust the finding of FMRFamide-like immunoreactivity associated with specific skeletal muscles in immunocytochemical studies (Myers and Evans, 1985*a*), together with the observation that radioimmunoassay of HPLC fractions demonstrates that muscle extracts have a similar immunoactivity profile to that of the thoracic nervous system, suggests that FMRFamide-like peptides may also be released onto these muscles to function either as neurotransmitters or as local

neuromodulators. Furthermore, radioimmunoassay of HPLC fractions of extracts from locust hearts and oviducts reveals the presence of some of the FMRFamide-like immunoreactive peaks found in nervous tissue as well as several peaks absent from nervous tissue. This raises the possibility that these visceral tissues may be innervated from the central nervous system by neurones containing FMRFamide-like peptides or, in the case of the heart, that peptides from this family may be associated with the intrinsic neurosecretory cells of the lateral cardiac nerve cord. It is likely that such neuronally released FMRFamide-like peptides would also play a role in the modulation of some aspects of the regulation of heart activity. Neurones containing FMRFamide-like peptides also innervate some molluscan non-cardiac muscles (Cottrell *et al.* 1983*b*) and both cardiac and non-cardiac muscles in the leech (Kuhlman *et al.* 1985*a*,*b*; Norris and Calabrese, 1987), where they have also been postulated to act either as neurotransmitters or as locally released neuromodulators.

The differential distribution in HPLC analysis of FMRFamide-like immunoreactive peaks in tissue extracts from different parts of the locust nervous system and potential target sites could be produced in a number of ways. Different precursor genes coding for FMRFamide-like peptides could be expressed differentially in the various cell types or the same single precursor gene could be activated in all the cell types, but its products could be processed differently in different tissues. We are currently attempting to sequence the other endogenous FMRFamide-like peptides and this information, together with the nucleotide sequence of the gene(s) encoding these peptides and the sequence of the mRNA(s) produced from it, will be required before a complete explanation of the control of the expression of FMRFamide-like peptides in this species can be obtained.

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