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Purification and Characterization of FMRFamidelike Immunoreactive Substances From the Lobster Nervous System: Isolation and Sequence Analysis of Two Closely Related Peptides

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ABSTRACT

In the preceding paper (Kobierski et al: *J. Comp. Neurol. 266*:1–15, '87) FMRFamidelike immunoreactivity (FLI) was localized to specific cells and processes in the nervous system of the lobster *Homarus americanus*. In an effort to establish a role for this material we have purified and characterized a variety of immunoreactive peptides that can be extracted from the secretory pericardial organs. By using gel-filtration chromatography and three different HPLC systems, it has been established that little or no authentic FMRFamide is present. Of the major immunoreactive components two peptides were purified in sufficient quantity for microsequence analysis and have been tentatively identified as the octapeptides Ser-Asp-Arg-Asn-Phe-Leu-Arg-Phe-amide (FLI 3) and Thr-Asn-Arg-Asn-Phe-Leu-Arg-Phe-amide (FLI 4). Both of these are novel neuropeptides with some sequence homology to the previously described FMRFamide family.

The pericardial organs release FLI when depolarized with 100 mM K^+ in the presence of calcium. Between 75 and 80% of this release is accounted for by FLI 3 and FLI 4. One of these peptides (FLI 4) has been synthesized and shown to cochromatograph with the endogenous immunoreactive material. Preliminary studies show that this peptide can act as a modulator of exoskeletal and cardiac neuromuscular junctions.

Key words: neuropeptides, hormones, crustacea

The cardioactive peptide FMRFamide (Phe-Met-Arg-Phe-amide) originally was isolated from the nervous system of the mollusc *Macrocallista nimbosa* (Price and Greenberg, '77), and an identical peptide has been found in two other molluscs, *Aplysia californica* (Lehman et al., '84) and *Helix aspersa* (Price, '82). The cDNA sequence corresponding to a mRNA that codes for FMRFamide in *Aplysia* also has been determined and shows that multiple copies of FMRFamide are likely to be contained within a single precursor polypeptide chain (Schaefer et al., '85; Taussig and Scheller, '86). More recently the gene that gives rise to multiple mRNAs containing FMRFamide coding sequences has been isolated (Taussig and Scheller, '86). In the search for FMRFamide-like peptides in the nervous systems of other species, a variety of immunologically related materials have been described (Boer et al., '80; Dockray et al., '81; Grimmelikhuijzen et al., '82; Jacobs and Van-Herp, '84; O'Donohue et al., '84; Sorenson et al., '84; Watson et al., '84; Dockray, '85; Kuhlman et al., '85a; Myers and Evans, '85; Marder et al., '87). Three peptides from this group that have been purified were shown to contain similar but not identical amino acid sequences. Each member of this putative family has the carboxy-terminal sequence -Arg-Phe-amide, which is the main antigenic determinant for most of the antibodies used in identifying FMRFamidelike antigens (Dockray and Williams, '83; O'Donohue et al., '84; Marder et al., '87). This also is the most critical region of the peptide for the pharmacological actions of the family (Greenberg et al., '81, '83; Koo et al., '82; Painter et al., '82). In addition this region

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distinguishes the FMRFamide group from the closely related pancreatic polypeptide family which is characterized by the C-terminal sequence -Arg-Tyr-amide (Lin and Chance, '74; Tatemoto et al., '82).

Using antibodies directed against the carboxyl terminus of FMRFamide, we have mapped the distribution of FMRFamidelike substances in the nervous system of the lobster *Homarus americanus* (Kobierski et al., '87). Several aspects of this distribution suggest that FMRFamidelike peptides may play important roles in a wide variety of physiological functions in lobsters. These include its presence in putative motor neurons; its highly ordered distribution in terminals around the optic, antennal, accessory, and olfactory neuropil regions of the brain; its appearance in fibers projecting to the stomatogastric ganglion; and its presence in a superficial dense plexus of varicosities in the sheath around the ventral nerve cord and also in the neurosecretory regions of the second thoracic nerves and their pericardial organs (Kobierski et al., '87).

Before these functions can be investigated further we felt it necessary to determine the precise chemical structure of the immunoreactive material. This is important since single species may contain several distinct FMRFamidelike peptides (O'Donohue et al., '84; Watson et al., '84; Price et al., '85), each of which may have different pharmacological properties (Painter et al., '82; Cottrell, '83; Price et al., '85). Our initial survey of FMRFamidelike immunoreactivity (FLI) in the lobster nervous system showed that the pericardial organs (PCOs) were the richest source (Kobierski et al., '87). Therefore we have used these structures as the starting point to try to purify and determine the chemical structure of *Homarus* FLI. We report here the isolation, purification, and sequencing of two major immunoreactive components of the PCOs. These are new peptides with some sequence homology to the previously described FMRFamide family. We also present preliminary evidence that these peptides can be released from PCOs in a calcium-dependent fashion by depolarization.

MATERIALS AND METHODS

Radioimmunoassay

The antibodies used in this study were generated in rabbits immunized with FMRFamide conjugated to succinylated thyroglobulin. Antibody 231 was a generous gift of Dr. Thomas O'Donohue at NIH. This serum has been well characterized and it displays a high affinity for peptides with the C-terminal sequence -Met-Arg-Phe-amide (O'Donohue et al., '84). The second antibody (671c) was the kind gift of Dr. R. Calabrese. This antibody has equal apparent affinities for Phe-Leu-Arg-Phe-amide and FMRFamide (Marder et al., '87). With both antibodies, 231 and 671c, the amidated carboxyl terminus is required for immunoreactivity. The RIA procedure using antibody 231 was that described in Kobierski et al. ('87). The same protocol was used with antibody 671c with the conditions described in Marder et al. ('87). Successive dilutions of crude FLI resulted in a linear increase in the amount of I^{125}YMRFamide binding to either antibody. The slopes of these lines were parallel to similarly generated FMRFamide dilution curves, indicating that the assay could be used to quantify *H. americanus* FLI. Samples were evaporated to dryness in a Speed-Vac vacuum concentrator prior to assay and then dissolved in RIA buffer. All values are reported as FMRFamide molar equivalents using antibody 231, unless otherwise stated.

Preliminary characterization of FLI

Sizing. Initial sizing of FLI was carried out on crude extracts by using small gel-filtration columns. Sephadex beads (G-25 Superfine) were swollen and washed in 1.25% acetic acid and packed into Bio-Rad minicolumns (10 mm i.d. × 100 mm). Dried extracts of two to eight PCOs were dissolved in 50 μl of 1.25% acetic acid containing 2 mg/ml Dextran blue and carefully layered on top of the gel bed. The separation was carried out by using 1.25% acetic acid as the mobile phase flowing at 7.5 ml/hour. The outflow of the column passed through an Altex model 153 U.V. absorbance detector set at 280 nm. The columns were calibrated by using Dextran blue (void volume), trypsin inhibitor, substance P, proctolin, FMRFamide, and tryptophan (total volume).

Trypsin degradation of FLI. Crude methanolic extracts of PCOs containing approximately 300 fmol (FMRFamide equivalents) of FLI were incubated for 1 hour at 37°C with 0.05 mg trypsin (type 1 from bovine pancreas—Sigma) in sodium phosphate buffer (pH 7.5). Control incubations were carried out in the presence of 0.05 mg trypsin inhibitor (type 11-S from soybean—Sigma). The reaction was stopped by cooling the tubes on ice and adding trypsin inhibitor to the experimental samples. Bovine serum albumen (0.05 mg) was then added and the immunoreactivity was assayed in the usual fashion. For comparison, samples of FMRFamide (600 fmol per sample) also were treated with trypsin in the absence or presence of inhibitor. Additional controls consisted of assaying trypsin (with and without inhibitor) and untreated samples of FLI or FMRFamide.

Oxidation of FMRFamide and FLI. Sixteen picomole equivalents of FLI and 2.8 nmol of FMRFamide were treated in HPLC solvent with 0.2% hydrogen peroxide for 140 minutes at room temperature. These solutions were injected directly onto HPLC columns (systems B and C) and the peaks were recorded by U.V. absorbance (FMRFamide) or by RIA of collected fractions (FLI). Control (nonoxidized) samples were injected immediately after adding the hydrogen peroxide. These mild oxidation conditions are sufficient to form the sulphoxide derivative of the methionyl residue in FMRFamide (Toennies and Callan, '39).

Peptide isolation and purification

Pericardial organs were dissected in cold saline from adult lobsters (*H. americanus*) weighing approximately 0.5 kg. The saline composition was that described in Evans et al. ('76). In general PCOs arising from the second, third, and fourth thoracic ganglia were used. Each pair of PCOs was extracted and stored in 200 μl of cold methanol/acetic acid (99:1 vol:vol). When sufficient organs had been accumulated they were ground to a fine suspension in a glass homogenizer and centrifuged at approximately 50,000g for 60 minutes. The supernatant fluid was collected and the pellet was reextracted in acidified methanol. This was centrifuged and the supernatant fluid was added to that from the first centrifugation. Aliquots of the supernatant solution and of the resuspended pellet were removed for protein determination and radioimmunoassay (RIA). The pooled supernatant fluids were dried at 60°C under a stream of dry nitrogen or by a vacuum centrifugation (Speed-Vac—Savant Instruments) after purging the centrifuge chamber with pure nitrogen. Wherever possible polypropylene or polycarbonate tubes were used since significant losses of purified immunoreactive material occurred on contact of

solutions with clean glassware. The dried extract was resuspended in 0.1% trifluoroacetic acid (TFA) by immersion in a sonicator bath (Laboratory Supplies Company, Inc.) for several minutes and then was subjected to a preliminary purification using small C_{18} reverse-phase cartridges (Sep-Pak, Waters Associates). The Sep-Pak matrix was activated with 5 ml acetone, washed with 5 ml water, and equilibrated with 5 ml of 0.1% TFA. The resuspended extract was applied in a volume of 6 ml/cartridge. Care was taken to use a sufficient number of cartridges to prevent overloading of the matrix (less than 1 mg protein was applied/cartridge). Hydrophilic substances were eluted from the cartridge with 5 ml 0.1% TFA, followed by 5 ml of 10% acetonitrile in 0.1% TFA. The FLI was eluted with 5 ml of 40% acetonitrile in 0.1% TFA. Finally, highly hydrophobic substances were rinsed from the cartridge with 80% acetonitrile in 0.1% TFA. Aliquots were removed from each of these fractions and dried before assaying. In later experiments the peptides were eluted with a single treatment of 5 ml of 60% methanol/0.1% trifluoroacetic acid/water (MeOH/TFA). Although recovery from the cartridges was similar for both procedures, better final yields were obtained upon drying samples from methanol than from acetonitrile.

High-performance liquid chromatography (HPLC). Three main HPLC systems were used in the characterization and purification of FLI. All of these systems used the same pumps (Water Associates 6000A and M-45), controller (Waters Associates Model 660 solvent programmer), injection valve (Altex 210), U.V. absorbance detector (Waters model 441, at 214 nm), and fraction collector (ISCO model 1850), but utilized different columns and mobile phases as described below.

HPLC A. In this system a C_{18} reverse-phase HPLC column (μBondapak. 39 × 300 mm—Waters Associates) was used at a flow rate of 0.5 ml/minute with fractions collected at 1-minute intervals. Nonhydrophobic substances were eluted isocratically in 0.1% TFA before beginning a linear gradient of acetonitrile ascending to 40% acetonitrile/0.1% TFA over 60 minutes. The solvents were degassed before use by filtration through a Nylon 66 filter (0.45 μm—Rainin Instrument Co. Inc) under reduced pressure.

HPLC B. In this second system another μBondapak column was used but the mobile phase consisted of 35% MeOH/0.1% TFA at a flow rate of either 0.5 or 1 ml/minute. The solvents for HPLC B were filtered and degassed through Nylon 66 filters before sparging with pure nitrogen. They were kept under nitrogen throughout the separation. This allowed the U.V. absorbance to be monitored at very high gain with minimal drift or background noise.

HPLC C. This system had the greatest resolving capability for the FLI peptides. The mobile phase consisted of 65% acetonitrile in 0.1% TFA in water pumped at a flow rate of 0.5 ml/minute. The column was a 250 mm × 4.6 mm Altex 10 μm Spherisorb C_{18} reverse-phase column that had been treated with sodium octyl sulphate and washed with MeOH, methylene chloride, and n-heptane before equilibrating with the mobile phase. The selectivity of this column was distinct from the other two reverse-phase systems, presumably because the matrix retained some charged groups, allowing a degree of ion-pairing to occur.

Microsequencing. The purified peptides eluting from the final HPLC step were dried in acid-washed polypropylene Eppendorf tubes and applied to the glass-fiber frit of a sequencer in MeOH/TFA. The derivatization and cleavage cycles were carried out on an Applied Bioscience Inc. 470A protein sequencer. The amino acid derivatives from each cycle were then separated and assayed by using a Hewlett-Packard 1090 HPLC system incorporating a Zorbax C_{18} reverse-phase column (DuPont) and a 1040 diode array detector system. We are grateful to Mr. William Lane of the Harvard Microchemistry Laboratory for performing this analysis.

Release of FLI from PCOs

FLI was released from isolated PCOs by depolarizing them with elevated potassium saline in the presence of Ca^{++}. In Ca^{++}-free saline the Ca^{++} was replaced by Mg^{++}. The osmolarity of the high K^+ (100 mM) saline was maintained by a corresponding decrease in the sodium concentration. In short time course experiments six organs from a single animal were dissected, placed into a small chamber, and washed thoroughly by passing 30 ml of saline through the chamber over 60 minutes. The organs then were incubated for 5 minutes in successive 0.05-ml aliquots of the appropriate solutions (see Results). To prevent differences in salt composition from influencing the RIA, each sample was desalted over a C_{18} Sep-Pak as described for the PCO extracts and the eluant was dried before reconstitution in RIA buffer for assay.

In some experiments FLI was collected over an extended period of time. In these cases the organs were placed in the chamber, which was perfused at 0.8 ml of saline per minute. The organs were washed with normal saline for 60 minutes before directing the effluent over an activated Sep-Pak and collecting any released peptides. The perfusing saline was then changed to one containing high K^+, and this effluent was directed over a second activated Sep-Pak. Cycles of high and low K^+ saline could be alternately applied to the organs and FLI contained within each perfusate was collected on a separate Sep-Pak. Ca^{++} was present in the saline in these experiments. Each Sep-Pak received the same total volume of perfusate from the PCOs. These incubations were carried out at 12°C. The collected peptides were eluted from the Sep-Paks for HPLC analysis.

Materials

HPLC-grade MeOH and acetonitrile were obtained from J.T. Baker Chemicals Co. and HPLC-grade water from American Burdick and Jackson. TFA (HPLC/Spectro grade) was obtained in 1-g sealed ampoules from Pierce Chemical Co. Acetic acid was from Fisher Scientific. FMRFamide and [D-Ala]^2-Met-enkephalin were supplied by Sigma Chemical Co. Eledoisin-related peptide (Lys-Phe-Ile-Gly-Leu-Met-NH_2) and angiotensin III (Arg-Val-Tyr-Ile-His-Pro-Phe) were obtained from Calbiochem. Proctolin and Tyr-Met-Arg-Phe-amide were from Peninsula Laboratories. Thr-Asn-Arg-Asn-Phe-Leu-Arg-Phe-amide was custom synthesized to greater than 99% purity by Neosystem Laboratories, France.

RESULTS

Isolation and characterization of FLI

Pericardial organs (PCOs) were used for the extraction and purification of the lobster FMRFamide-related peptides, because of their high content of FLI (approximately 2-pmol equivalents per organ) (Kobierski et al., '87) and because of the relative homogeneity of the tissue (mostly neurosecretory terminals). We explored several different methods of extracting FLI from the tissue. Equal amounts of FLI were recovered by extracting PCOs in acidified meth-

Abbreviations

FLI	FMRFamidelike immunoreactivity
FLI 3	Ser-Asp-Arg-Asn-Phe-Leu-Arg-Phe-amide
FLI 4	Thr-Asn-Arg-Asn-Phe-Leu-Arg-Phe-amide
HPLC A	Column-μBondapak C_{18} reverse phase; Mobile Phase-0.1% TFA followed by linear gradient of ascending acetonitrile to 40% acetonitrile/0.1% TFA
HPLC B	Column-μBondapak C_{18} reverse phase; Mobile phase-35% methanol/0.1% TFA
HPLC C	Column-Altrex 10 μm Spherisorb C_{18} reverse phase treated with sodium octyl sulphate and washed with methanol, methylene chloride, and n-heptane; Mobile phase-65% acetonitrile/0.1% TFA
MeOH/ TFA	methanol/0.1% trifluoroacetic acid/water
PCO	pericardial organ
RIA	radioimmunoassay
TFA	trifluoracetic acid

anol or in RIA buffer containing the protease inhibitors bacitracin and phenylmethylsulphonylfluoride (results not shown). Acetone also extracts FLI from the tissue but the yields are lower and less consistent. These results suggested that the major portion of the immunoreactive material was not denatured by organic solvents and that FLI consisted of relatively small or relatively polar molecules that are capable of dissolving in solvents of moderately low dielectric constant. Because acidified MeOH precipitates many large proteins and inactivates most enzymes, it was used as the primary extraction procedure throughout the experiments reported here.

Like FMRFamide, the immunoreactive material was found to be susceptible to degradation by proteolytic enzymes. Incubation with trypsin destroyed 84% of FMRFamide and 93% of the FLI. The degradation was quantitatively inhibited by soybean trypsin inhibitor. Initial sizing experiments using small Sephadex G-25 gel filtration columns showed, however, that lobster FLI contained relatively little or no authentic FMRFamide. Crude methanolic extracts of the tissue applied to columns showed a broad peak of immunoreactivity eluting after the void volume but before authentic FMRFamide (data not shown). A low recovery from these columns (20%), however, discouraged the use of this procedure as an initial purification step.

Partial purification with better recovery was achieved by applying crude extracts to Sep-Pak C_{18} reverse-phase cartridges. Salts and other hydrophilic materials were eluted with 0.1% TFA and then the immunoreactive material was eluted with acetonitrile or MeOH. The recovery of FLI was greater than 80%. This partially purified extract was characterized further on several reverse-phase gradient elution HPLC systems (Fig. 1A–C). On system A no immunoreactivity was detected during the isocratic polar elution phase of the chomatogram but three peaks of FLI were resolved during the nonpolar gradient (Fig. 1A): the earliest peak (18% acetonitrile) eluted close to FMRFamide and comprised 15% of the recovered immunoreactivity; the major peak (23% acetonitrile) contained 56% of the recovered FLI; and a small broad peak eluting late in the gradient contained approximately 29% of the recovered material. No measurable FLI appeared in later fractions. The overall recovery from these columns was between 60 and 90%. With the two isocratic systems (B and C) further peaks of

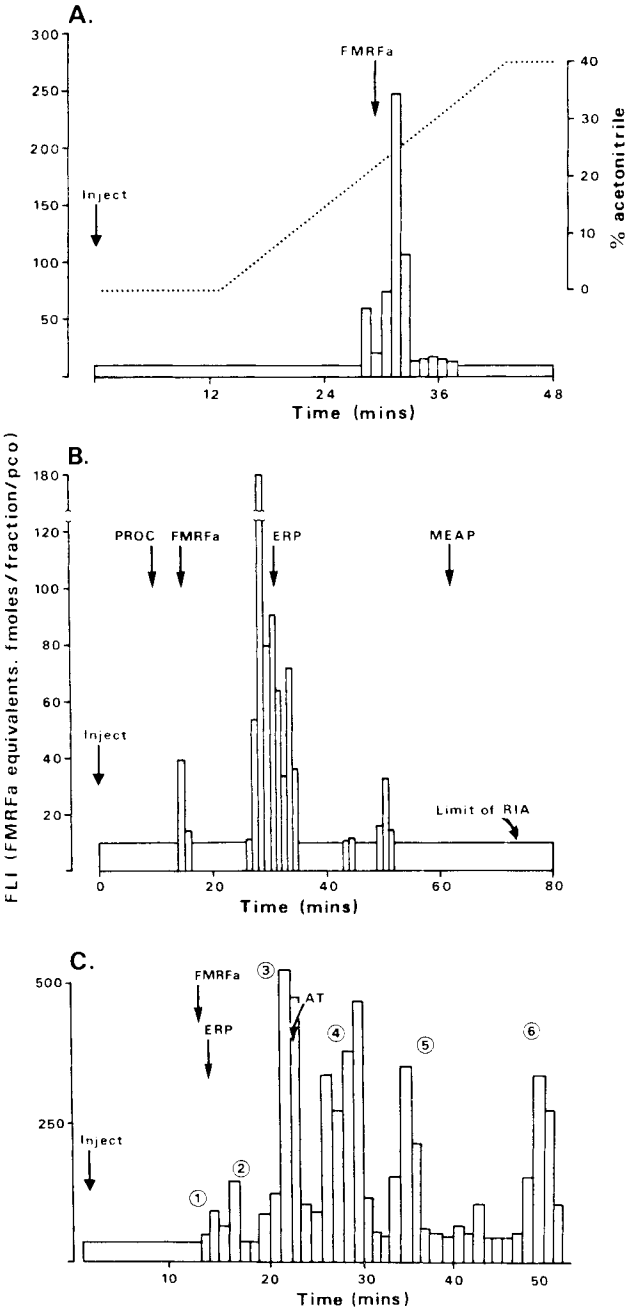

Fig. 1. HPLC characterization of partially purified FLI. **A:** An extract of six PCOs was injected onto HPLC system A at the point shown. After a 13-minute elution with 0.1% TFA, a gradient of increasing acetonitrile was begun, as shown by the dotted line (scale on the right). Fractions were collected at 1-minute intervals and after drying assayed for FLI. **B:** An extract of six PCOs was injected onto HPLC system B and fractionated isocratically in 35% MeOH/0.1% TFA. The elution positions of proctolin (Proc), FMRFamide (FMRFa), eledoisin-related peptide (ERP), and Met-enkephalin-Arg^6-Phe^7 (MEAP) are indicated. **C:** A similar extract of two PCOs was injected onto HPLC system C and fractionated in 65% acetonitrile/0.1% TFA. Fractions collected at 1-minute intervals were assayed after drying. The elution positions of FMRFamide (FMRFa), eledoisin-related peptide (ERP), and angiotensin III (AT) are indicated. The peaks are numbered according to increasing retention time and are identified on the basis of many such separations. The amount of FLI is represented as fmol/fraction/PCO by using antibody 231 (A and B) or antibody 671c (C). The peaks were qualitatively identical with either antibody (see text).

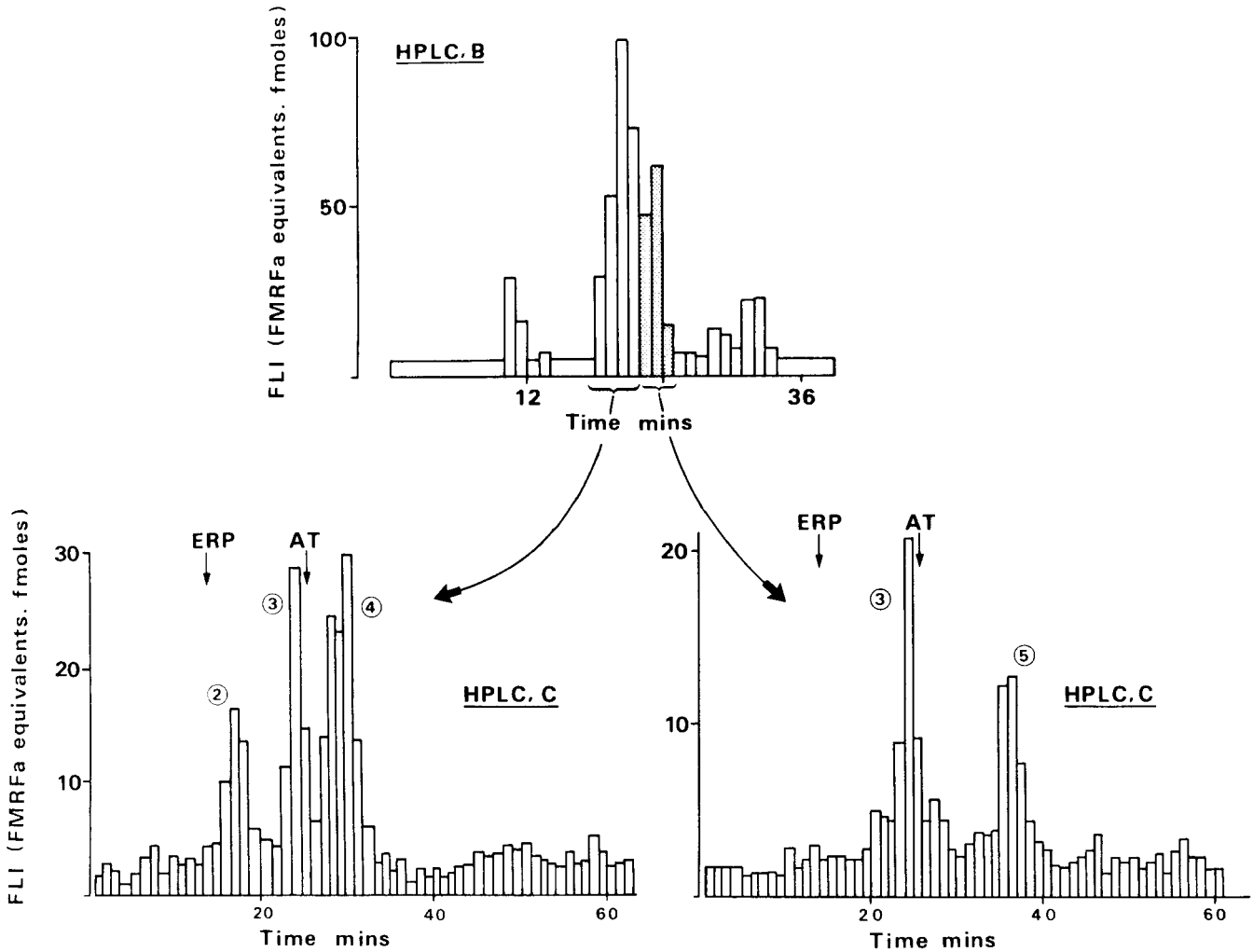

Fig. 2. The identification of several immunoreactive components within the main peak of FLI from HPLC B. The upper figure shows the elution profile of immunoreactivity from HPLC B. The main peak was divided into two samples by pooling the earlier eluting fractions (open bars marked by braces) and likewise the late fractions (stippled bars). These two samples were separately injected onto HPLC C and the resulting immunoreactive profiles are illustrated in the lower two figures. Peaks are numbered by comparing their retention times with peaks identified in whole extracts (Fig. 2, HPLC, C). The elution positions of eledoisin-related peptide (ERP) and angiotensin III (AT) are indicated.

FLI were observed. On system B four peaks were resolved (Fig. 1B) and on the other (C) at least six separate bands appeared. For convenience the peaks were numbered in system C from 1 to 6. This does not imply that each peak contains a single component but allows comparisons to be made between the different HPLC systems and facilitates reference to distinct immunoreactive fractions.

The relationship between the different FLI peaks was investigated by subjecting the main immunoreactive peak of HPLC A to sequential fractionation on systems B and C (Fig. 2). This fraction gave rise to two peaks on HPLC B (the major and late peaks). The major peak of HPLC B was resolvable into four of the peaks on the HPLC C (2–5). The early part of the B peak contained fractions 2–4 of system C, while the late part contained fractions 3 and 5 (see Fig. 2).

This multiplicity of immunoreactive peaks raises two possibilities: (1) that there is a large family of FMRFamide-related peptides in lobsters or (2) that several peptide forms are generated from a single "parent" peptide during the purification procedure. FMRFamide itself changes its chromatographic properties according to the oxidation state of the methionyl residue. To investigate the possibility that some of the peaks we observe might be oxidation products of a parent peptide, samples of partially purified FLI were fractionated by HPLC system C after treatment with hydrogen peroxide. The elution position and number of peaks were unaffected by oxidation conditions sufficient to completely oxidize 500 ng of FMRFamide, but the proportions of immunoreactivity eluting in the various peaks changed slightly (data not shown). A similar result was obtained with HPLC B, which separates FMRFamide and its sulphoxide by a wide margin. The early part of the major immunoreactive peak increased by approximately 10% (results not shown). This effect is discussed later. The general lack of effect of hydrogen peroxide indicates that the majority of FLI is not susceptible to mild oxidation. Either the peptides are already oxidized or they lack a methionyl residue.

Further evidence for the lack of a methionyl residue was obtained by comparing the relative affinity of two antibody preparations for partially purified FLI. Antibody 231

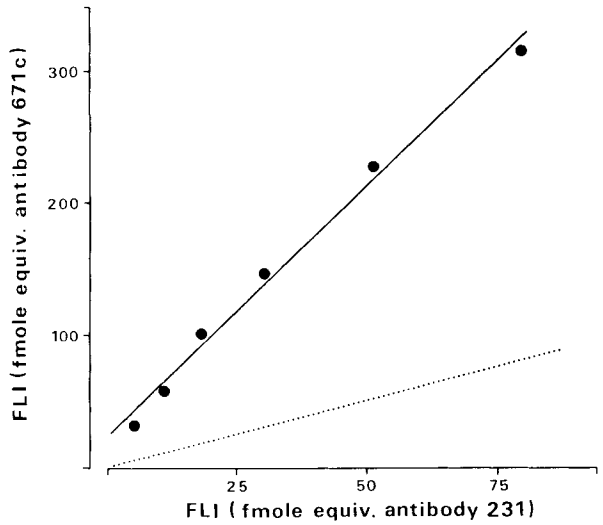

Fig. 3. The affinities of the two antibodies used in the present study for *Homarus* FLI and for FMRFamide. A sample of partially purified extract was serially diluted and assayed using the two different antibodies. The standard curves for each assay were constructed from identical samples of FMRFa and the FLI values were interpolated from them. Each point is the average of duplicate determinations using each antibody. The regressed line has slope of 3.9. The dotted line has a slope of 1.0 and indicates the expected regression line for antibodies having the same affinity for FLI relative to FMRFa. Antibody 671c gives a fourfold-higher molar estimate of FLI than antibody 231.

(O'Donohue et al., '84) consistently underestimated the amount of FLI detected by antibody 671c (Marder et al., '87) (Fig. 3). Antibody 671c has an equal affinity for FMRFamide peptides with leucine or with methionine at the 2-position (Marder et al., '87) but antibody 231 has a higher affinity for peptides with a methionine residue at this position (O'Donohue et al., '84). We suspect therefore that the major components of lobster FLI have no methionine within the antigenic determinants.

While we have not investigated many different experimental protocols for extraction and separation of the FMRFamide-related peptides, we think it unlikely that the large number of peptides is an artifact of the separation procedure. Each peak of immunoreactive material can be collected and rechromatographed on the same or either of the other two systems with a characteristic and stable retention time.

Purification and identification

To collect sufficient material for sequence analysis of these immunoreactive peptides, 240 PCOs from 50 lobsters were extracted and desalted over Sep-Paks. FLI was eluted and applied to HPLC B. The major immunoreactive peak from HPLC B was then fractionated on HPLC C. A narrow range of fractions from the center of each immunoreactive peak was then reapplied to HPLC B and fractions were collected. Two of those contained immunoreactivity exactly coincident with an absorbance peak (3 and 4 from HPLC C; Fig. 4), suggesting that they might be present in sufficient amounts for microsequencing. From the 800 pmol (FMRFamide equivalents) extracted from the PCOs we obtained approximately 40 pmol of each of the purified peptides FLI 3 and FLI 4. Cycles 5 and 6 of the solid-state microsequencing procedure are shown for peak 3 in Figure 5. The amino acids found were phenylalanine (cycle 5) and leucine (cycle 6) at yields of approximately 20 pmol. With such low levels of amino acid, the sequencing apparatus must be run at maximum sensitivity, resulting in several problems in the interpretation of the data. For example, in the first two cycles (the amino terminus) even tiny amounts of contaminating free amino acids or other amines in the solvents or samples can obscure the sequence. In addition, tryptophan, cysteine, and methionine are extremely difficult to detect with the Edman sequencing method. What we present in Table 1 therefore are the most likely sequences deduced by matching the amino acid yields at each cycle. Possible alternative amino acids cannot be ruled out completely. In all these analyses we detected no amino acid derivatives after cycle 8, which indicates that these peptides contain only eight (or fewer) amino acids.

The sequence data for peak 4 suggested that a mixture of two peptides was present. In each cycle (except cycle 8) of the sequencing of peak 4 we detected two amino acid derivatives (Table 1). The appearance of each amino acid at two successive steps is consistent with the notion that two peptides are present, each sharing a common seven carboxy terminal amino acids and with one peptide having an extra amino terminal threonine (see ascribed sequences, Table 1).

Confirmation of the peak 4 sequences by peptide synthesis

To test that the proposed sequence of peak 4 was correct, the octapeptide Thr-Asn-Arg-Asn-Phe-Leu-Arg-Phe-amide (Thr-8-Phe-amide) was custom synthesized to high purity and samples were injected onto HPLC systems B and C. To make direct comparisons with lobster FLI fresh extracts from PCOs were fractionated on each system prior to running the peptide. The synthetic peptide coeluted with the major immunoreactive peak of system B (results not shown) and with the early part of peak 4 in system C (Fig. 6). We also have preliminary results indicating that the septapeptide formed after cleaving the amino-terminus threonine from the synthetic peptide elutes in the later part of peak 4.

Both antisera used in this study were capable of binding to the synthetic peptide (Fig. 7) but with lower apparent affinities than for FMRFamide itself. However, the affinities of serum 671c for FMRFamide and Thr-8-Phe-amide were much less widely separated, so this serum will give a closer molar estimate of the endogenous peptide when FMRFamide is used as a standard. Interestingly, the apparent affinities of serum 671c for FMRFamide and Thr-8-amide in the present study (B/Bo = 0.5 at 1.04 nM and 3.25 nM, respectively) are the same as those reported for FMRFamide and *Helix* peptide (Marder et al., '87).

The release of FLI

Isolated PCOs could be stimulated to release FLI into the bathing medium by depolarizing them with 100 mM K^+ in the presence of 2.6 mM Ca^{++} (data not shown). Elevated K^+ would not increase the release of FLI if Ca^{++} was replaced with Mg^{++}. There was a persistent but declining spontaneous release of FLI when the organs were maintained in ordinary saline (not shown). The level of this background release was variable from experiment to experiment and it may arise through leakage from damaged axons and terminals. High K^+ saline in the presence of Ca^{++} about doubled the efflux of FLI (six experiments). The amount released during a 5-minute treatment with high K^+ saline was less than 0.4% of the immunoreactive material found in the PCOs.

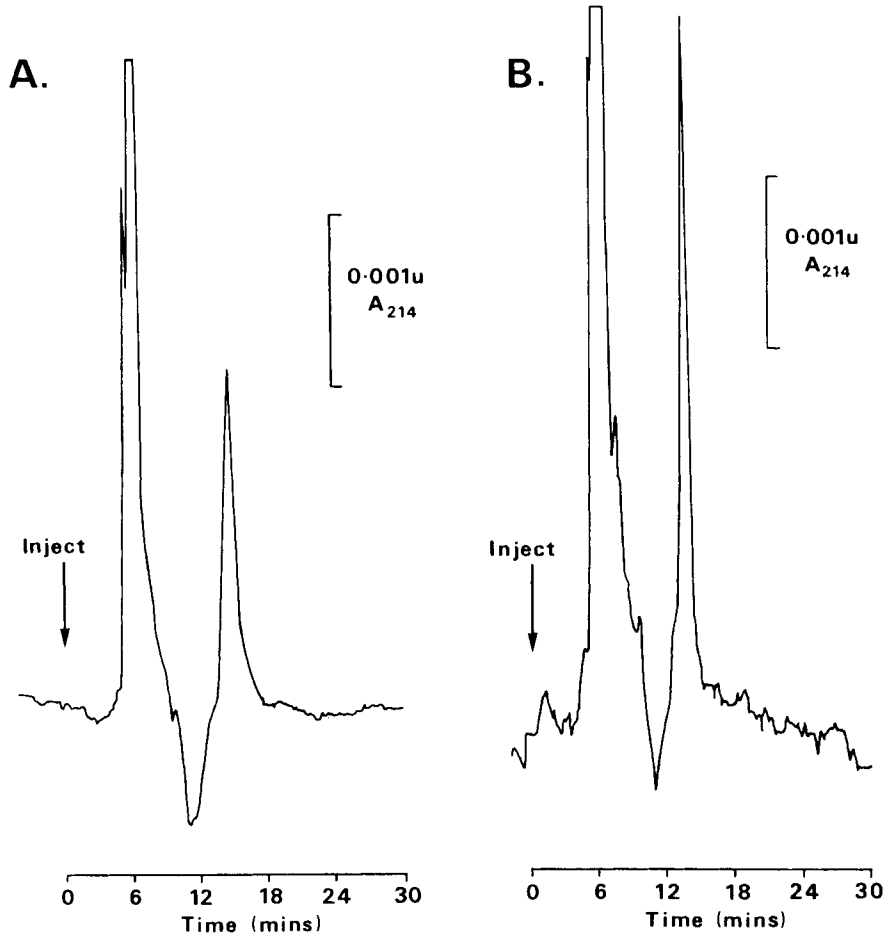

Fig. 4. High-gain U.V. absorbance (214 nm) profiles of the purified FLI peaks 3 and 4. Each peak is associated with all of the eluted immunoreactivity. The peak area of **A** corresponds to approximately 45 pmol (with angiotensin III as a calibration standard) and that of **B** is 35 pmol. Each of these samples was used for sequencing.

To collect sufficient FLI for HPLC analysis, released material was accumulated on a Sep-Pak by using the long-term protocol described in Materials and Methods. Alternate cycles of high and low K^+ salines were passed over separate Sep-Paks. The material collected during both the high and low K^+ perfusion was fractionated on HPLC system C. All six of the resolvable peaks were elevated over background levels by depolarization (Fig. 8). However, between 75 and 80% of the increase in release caused by high K^+ was accounted for by peaks 3 and 4 alone. It is interesting that the material in peak 4 eluted only in the early part of the normal peak 4 span (compare Fig. 8 with Figs. 1 and 2). This observation is consistent with the release of the single eight amino acid peptide of peak 4 (see Fig. 6) and suggests that the seven amino acid peptide might be a proteolytic product formed during tissue extraction.

DISCUSSION

We have isolated and determined the amino acid sequences of several FMRFamide-related peptides from extracts of lobster pericardial organs, a well-known crustacean neurohemal structure. While the antibodies used to detect and quantitate these peptides indicate that -Arg-Phe-NH_2 is the carboxyl-terminal sequence (O'Donohue et al., '84; Marder et al., '87), none of the isolated peptides is identical to authentic FMRFamide. All of the peptides characterized thus far are larger than FMRFamide and all have a leucine substituted for the methionine of FMRamide. The terminal four amino acids of the lobster peptides (-Phe-Leu-Arg-Phe-NH_2) are found in septapeptides isolated from the snail (Price et al., '85), while the terminal three amino acids are identical to those of a chicken brain FMRFamidelike peptide (Dockray et al., '83). The sequence of amino acids at the amino termini of the lobster peptides, however, differs substantially from the amino termini of previously identified FMRFamidelike peptides. In addition, the lobster sequences do not correlate with any nucleotide sequences found in the *Aplysia* gene for the FMRFamide precursor protein (Schaefer et al., '85: Taussig and Scheller, '86). The lobster peptides, therefore, may be representatives of a unique crustacean family of FMRFamide-related peptides (see below).

Several lines of evidence indicate that these are a true family and not artifacts of the extraction or isolation procedures. The fact that they are amidated indicates that they are formed from precursor molecules relatively late in the processing sequence. Moreover, the release experiments show that all six peaks of FLI identified with HPLC system C are elevated in the medium on stimulation, although most of the released peptides are the forms found in peaks 3 and 4. We presume that the small generalized release of FLI into normal saline is caused by a nonspecific, perhaps

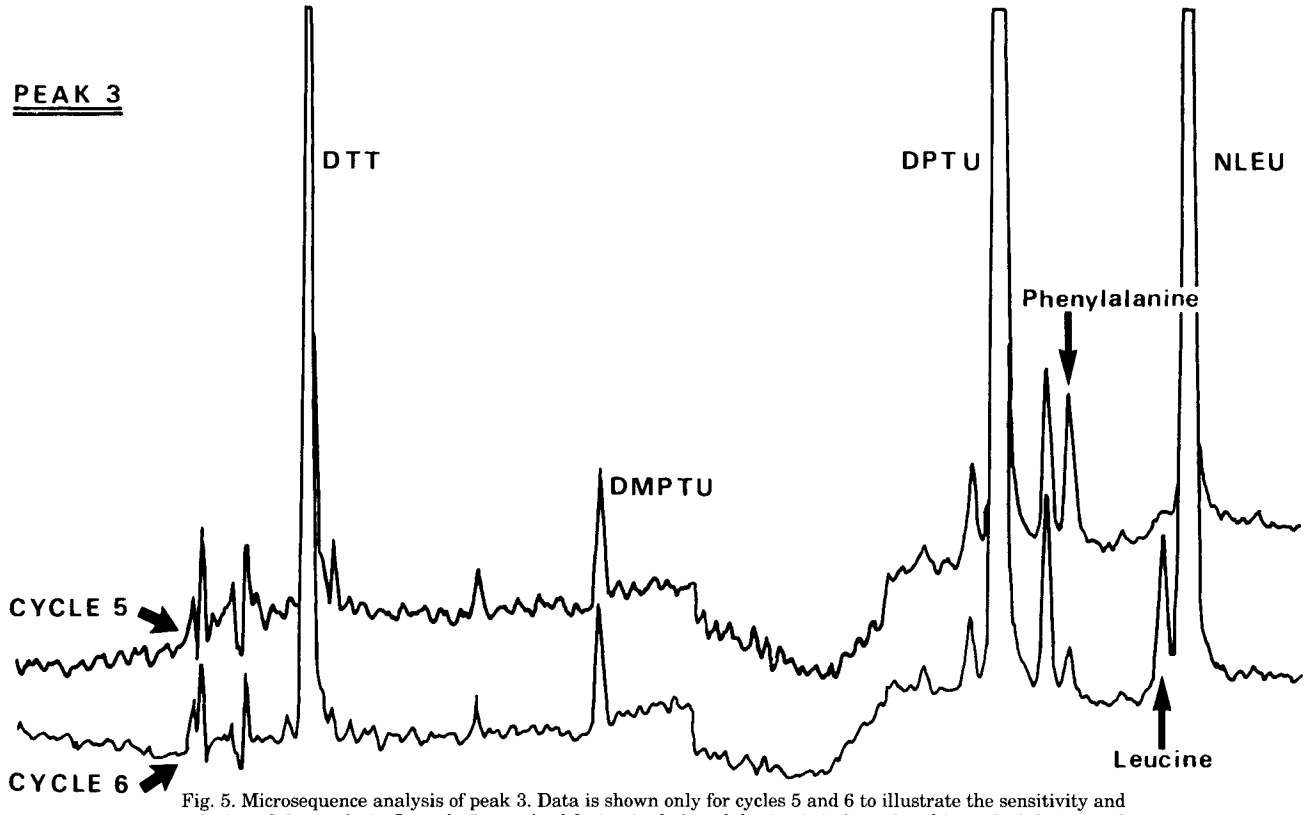

Fig. 5. Microsequence analysis of peak 3. Data is shown only for cycles 5 and 6 to illustrate the sensitivity and resolution of the analysis. In cycle 5 a peak of derivatized phenylalanine is indicated and in cycle 6 derivatized leucine is clearly visible. The positions of standards used in calibrating each run are also shown. Dithiothreitol (DTT), dimethylphenylthiourea (DMPTU), diphenylthiourea (DPTU), and norleucine (NLEU).

TABLE 1. Amino Acid Sequence Data for Peptides in Peaks 3 and 4[1]

	Microsequence cycle							
	1	2	3	4	5	6	7	8
Peak 3	S (G) (F)	D	R	N	F	L	R	F
Ascribed sequence (FLI 3)	S	D	R	N	F	L	R	F
Peak 4	(T) N	N R	R N	N F	F L	L R	R F	F
Ascribed sequences (FLI 4a)	T	N	R	N	F	L	R	F
(FLI 4b)	N	R	N	F	L	R	F	

[1]The amino acids identified at each cycle of the microsequence analysis are shown for peak 3 and peak 4 peptides. For peak 3 the first cleavage cycle produced three amino acids. Yield-matching indicated that serine was the most likely choice but glycine and phenylalanine are other possibilities. For peak 4 two amino acids were cleaved at each cycle. Yield-matching and the out-of-phase appearance of each residue are consistent with the presence of a seven amino acid (cleaved) version of FLI 4a. The yield of threonine in cycle 1 was low and is thus tentatively identified as the first residue. Abbreviations: S = Ser; D = Asp; R = Arg; N = Asn; F = Phe; L = Leu; G = Gly; T = Thr.

disruptive, effect of high potassium treatment and that the preferential release of peaks 3 and 4 represents the "normal" calcium-dependent release. In the absence of further information about the source of the minor FLI components, the possibility remains that they are breakdown products of the major peptides. The stimulated release of these major peptides could therefore lead to the small increases seen in the minor peaks. The observation that only the octapeptide of peak 4 is released by depolarization while a septapeptide is found in this peak in tissue extracts raises the possibility that some proteolytic degradation can occur during extraction. Oxidation of extracts with peroxide did not cause more than a 10% change in the size or location of any of the peaks seen on HPLC system C. This suggests that methionine is not a major constituent of the peptides extracted from this tissue. It remains possible that side-chain substitutions (carbohydrate, phosphate, sulphate) could account for some of the multiplicity of peaks we observe (Mains et al., '83; Nachman et al., '86). Finally, we expect, if anything, that more rather than fewer FMRFamide-related peptides or peptide precursors will be isolated from lobster tissues in the future, as the antibodies we have used require an amidated carboxyl terminus. Nonamidated peptides or larger, possible precursor, peptides therefore will have have been undetectable with the present protocol. The large number of FMRFamidelike peptides found in lobster tissue is remi-

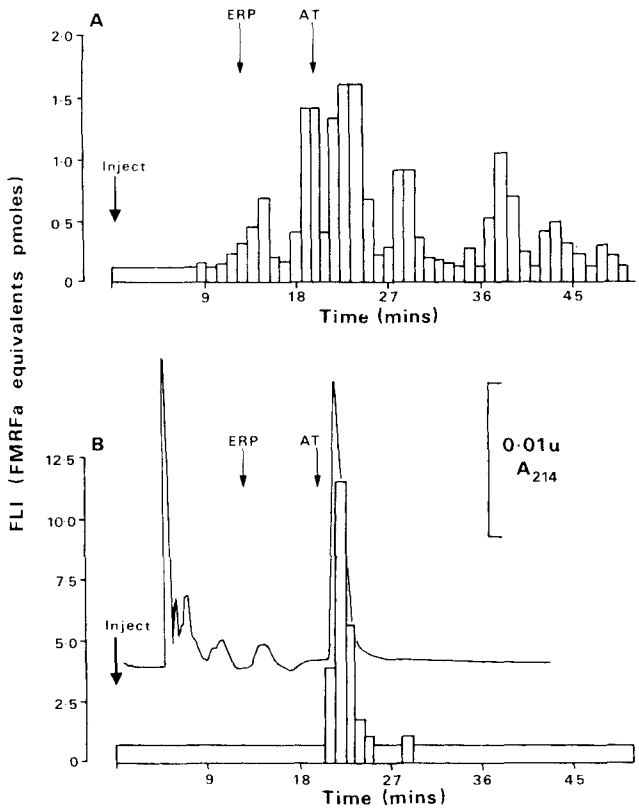

Fig. 6. Thr-8-Phe-NH_2 coelutes with the early part of peak 4. **A:** A sample of partially purified PCO extract was fractionated on HPLC as described in the Materials and Methods. **B:** The eluted immunoreactivity after applying 30 pmol of Thr-8-Phe-NH_2, to the same system. The peptide exactly coelutes with the early part of peak 4. This immunoreactivity corresponds to an U.V. absorbance (214 nm) peak that is shown here after injecting 1 μg of the peptide.

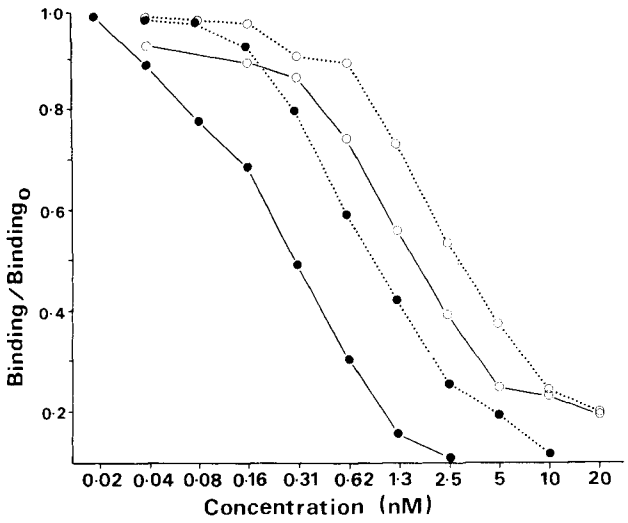

Fig. 7. Comparing the affinities of antibody 231 and 671c for FMRFamide and Thr-8-Phe-NH_2. The inhibition of radiolabel binding is represented as the proportion bound at a particular concentration of peptide (B) relative to that bound in the absence of peptide (B_0). Solid lines, antibody 231; dotted lines, antibody 671c. Filled circles are values for FMRFa and open circles for Thr-8-Phe-NH_2.

niscent of the very large family of opioid peptides isolated from vertebrate species. This raises further interesting similarities between the FMRFamidelike peptides of invertebrates and the opioid peptide families of vertebrates.

The role of these peptides in the lobster is at present unknown. FMRFamide exerts a variety of effects in invertebrate and vertebrate systems. When applied to specific *Helix* neurons both FMRFamide and an homologous opioid peptide, YGGFMRFamide, increase K^+ conductances and reduce a Ca^{++}-activated K^+ current (Cottrell, '82, '83; Cottrell and Green, '84). FMRFamide also may cause Na^+ conductance increases in some *Helix* neurons (Cottrell, '83; Boyd and Walker, '85) and an unidentified conductance increase in sensory neurons of *Aplysia* (Ocurr and Byrne, '85). Similarly, FMRFamide has actions on the membrane conductances of mammalian neurons (Gayton, '82; McCarthy and Cottrell, '84). One of the best characterized actions of FMRFamide is its ability to modulate the heartbeat of the leech (Kuhlman et al., '85b; Greenberg and Price, '83). Interestingly, FMRFamide analogues influence blood pressure when injected into the rat (Barnard and Dockray, '84; Wong et al., '85).

A limited number of physiological and chemical studies have been carried out with FMFRamide-related peptides in other species of Crustacea. In *Cancer borealis* FLRFamide is a more potent modulator of the stomatogastric pyloric rhythm than FMRFamide (Marder et al., '86). Moreover, there is evidence that the nervous system of *C. borealis* contains FLI that cochromatographs with peaks 3 and 4. The nerve cord of *Panulirus interruptus* contains a single FLI that cochromatographs with peak 4 (Marder et al., '87). These species therefore may process closely related or identical peptides to those found in *H. americanus*. These observations further support the existence of a unique crustacean branch of this most interesting set of related peptides found in species as diverse as *Aplysia* and man. The localization of FMRFamide-related immunoreactive substances to the stomatogastric ganglia of *P. interruptus*, *C. borealis*, and *H. americanus* (Marder et al., '87), in addition to their presence in the neuropil of the ventral nerve cord of *H. americanus*, points toward a synaptic role, possibly as a modulator for these compounds. Synthetic peak 4 of the lobster FLI increases the force and rate of contraction of the heart and causes contractions and increases the amplitude of excitatory junctional potentials in the dactyl opener muscle in *H. americanus* at 10^{-9} to 10^{-10} M threshold concentrations (Goy, Kravitz, and Trimmer, unpublished observations). Both effects can be elicited by FMRFamide, but at much higher concentrations (10^{-6} to 10^{-5} M). Boyd and Walker ('85) have demonstrated that in certain *Helix* neurons, substitution of the methionine in FMRFamide by leucine can in itself alter the physiological effect of the peptide. Price et al. ('85) note that a longer form of a *Helix* peptide (pQDPFLRFamide) is 100 times more potent on *Helix* heart muscle than FMRFamide. In addition, the effects of the longer peptide on tentacular and pharyngeal muscles are markedly different from those of FMRFamide (Cottrell et al., '83). Price has suggested that in addition to the different pharmacological effects of peptides containing an amino-terminus "tail," the amino acid extension could serve to protect the peptide from proteolytic degradation. This could allow the peptide to act as a neurohormone and to exert its effect at a considerable distance from its release sites (Price, '82; Price et al., '85). Possibly the amino-terminal extensions enable lobster peptides to persist in the the hemolymph as well, but until more is known about the specificity of lobster peptidases, this is conjectural.

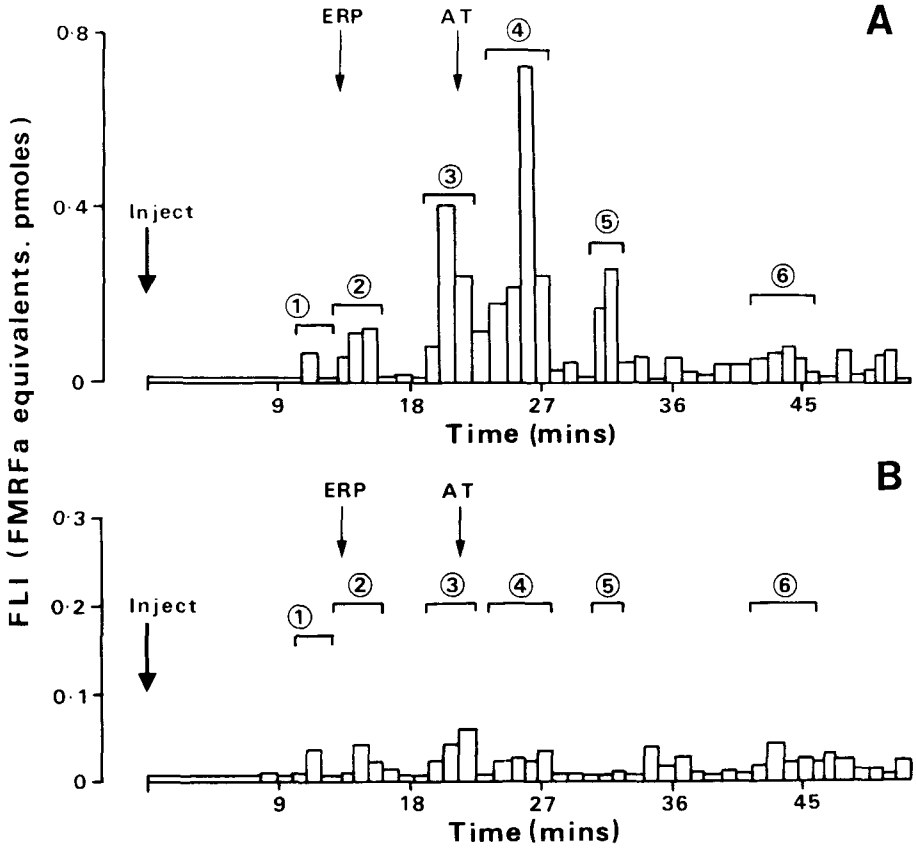

Fig. 8. The preferential release of FLI 3 and 4 by high potassium saline. FLI released during periods of 100 mM potassium treatment (A) and control saline (B) were fractionated on HPLC system C. Fractions were collected, dried, and assayed as described in the text. The elution positions of FLIs 1–6 and the marker peptides eledoisin-related peptide (ERP) and angiotensin III (AT) are indicated. Note that in B the vertical scale has been expanded relative to A. All values are FMRFamide equivalents using antibody 671c.

Much work remains to be done with the lobster FMRFamide-related peptides. While the present studies represent only first steps in exploring the role of these materials, the availability of pure synthetic peptides should allow careful physiological studies to be performed, and the sequence data could provide an important entry into molecular genetic studies with these interesting compounds.

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