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LOCALIZATION OF FMRFamide-LIKE PEPTIDES IN THE SNAIL *HELIX ASPERSA*

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SUMMARY

The distribution of FMRFamide-like material in the gastropod mollusc, *Helix aspersa*, was studied by radioimmunoassay (RIA) and immunocytochemistry. Most of the RIA activity was concentrated in the central nervous system, the male reproductive tract, the tentacles and the posterior digestive system (Table 1). The density of FMRFamidergic perikarya, nerves and nerve varicosities in the muscle tissue of all these regions, as indicated immunocytochemically (Fig. 2), was well correlated with the distribution as determined by RIA.

Gel chromatography of each extract resolved two peaks of FMRFamide-like immunoreactivity (Fig. 3). The first of these was further analysed by high-pressure liquid chromatography (HPLC), and the components included two major immunoreactive peaks identifiable, both by their retention times and their effects on the radula protractor muscle of *Busycon contrarium*, as the known peptides FMRFamide and pQDPFLRFamide (Figs 4–6). The second peak from gel chromatography gave only a single peak, distinct from that of FMRFamide and pQDPFLRFamide, in two HPLC systems (Fig. 7), but it did not behave like a competitive ligand in the FMRFamide RIA. Moreover, its immunoreactivity, unlike any peptides we tested, was not affected by carboxypeptidase Y (Fig. 8), and it was not active on the radula protractor muscle. Thus, it is certainly not an FMRFamide-like peptide.

We conclude that *Helix aspersa* contains at least two FMRFamide-like peptides, FMRFamide and pQDPFLRFamide. These peptides appear to act both as neurohormones and as neurotransmitters or modulators in the central ganglia, reproductive, digestive, muscular and circulatory systems.

INTRODUCTION

Cardioactive substances other than the classical transmitters (acetylcholine, 5-hydroxytryptamine and the catecholamines) have been detected frequently in molluscan ganglia (Frontali, Williams & Welsh, 1967; Kerkut & Laverack, 1960;

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Jaeger, 1966; Lloyd, 1978; Geraerts *et al.* 1984). Among these compounds is the family of FMRFamide-like peptides. FMRFamide itself was first isolated and sequenced from a clam, *Macrocallista nimbosa*, but has since been found in a number of gastropod species, including *Aplysia brasiliana*, *Lymnaea stagnalis* and *Pomacea paludosa* (Price & Greenberg, 1977; Lehman, Price & Greenberg, 1984; Price, 1986). *Helix aspersa*, a stylommatophoran pulmonate snail, contains FMRFamide, the heptapeptide pyroGlu-Asp-Pro-Phe-Leu-Arg-Phe-NH_2 (pQDPFLRFamide) and other related peptides (Price *et al.* 1985).

The actions of FMRFamide-like peptides have been well studied on a variety of molluscan muscles. First, the heart of *Mercenaria mercenaria* and the radula protractor muscle of *Busycon contrarium* have been ideal assay tissues due to their sensitivity, specificity and selectivity (Greenberg, 1983). In addition, the effects of FMRFamide have been surveyed on: a vast collection of bivalve hearts (Painter & Greenberg, 1982); the *Mercenaria* rectum (Doble & Greenberg, 1982); the gill, anterior gizzard and various neurones of *Aplysia* (Weiss *et al.* 1984; Austin, Weiss & Lukowiak, 1983; Stone & Mayeri, 1981); the auricle, ventricle, oesophagus and penis retractor muscle of *Lymnaea* (Geraerts *et al.* 1984); the octopus heart (Wells, 1983); and the *Helix* tentacle muscle (Cottrell, Greenberg & Price, 1983*a*; Cottrell, Schot & Dockray, 1983*b*). Finally, the cellular mechanisms of action of FMRFamide have been investigated, primarily with clam hearts (Higgins, Price & Greenberg, 1978), the anterior byssus retractor muscle of mytilid mussels (Painter, 1982), and specific neurones of *Helix aspersa* (Cottrell *et al.* 1983*b*).

In summary, FMRFamide affects all types of molluscan excitable tissue: heart, non-cardiac muscle and nerve. Notwithstanding the long list of its pharmacological actions, the physiological role of FMRFamide remains unknown. One hypothesis is that the peptide plays a cardioregulatory role in bivalves. This notion is based upon its cardioactive properties, its presence in the heart, and its presence in the blood (Greenberg & Price, 1980; Nagle, 1982). However, although FMRFamide has pronounced effects on the molluscan myocardium, other physiological sites of action have been proposed (Greenberg & Price, 1980). For example, FMRFamide may be the transmitter or modulator at particular neuromuscular junctions (Cottrell *et al.* 1983*b*; Austin *et al.* 1983).

As an approach to learning the physiological role of FMRFamide, this report describes the localization of FMRFamide-related immunoreactivity in the organs and tissues of *Helix aspersa*, while in the subsequent paper (Lehman & Greenberg, 1987) the distribution is correlated with pharmacological responsiveness and known functions. A radioimmunoassay for FMRFamide was used to determine the concentrations of FMRFamide-related activity in 23 tissues of *Helix*. Immunocytochemical methods were employed to examine the density of innervation, the types of cells innervated, and the locations of FMRFamidergic cell bodies; i.e. the probable sites of production of these peptides. The FMRFamide-like peptides of this snail were characterized by their elution volumes in gel chromatography and HPLC, and by their biological activity on the radula protractor muscle of *Busycon contrarium*.

MATERIALS AND METHODS

Animals

Helix aspersa were collected near Fullerton, California and sent by express mail to Florida. The snails were aestivating upon arrival, and they remained in this condition until dissected (anatomy described by Leake, 1975; Hyman, 1967).

Extraction

The snails were processed in groups of 30–100. The first step was to take blood through a syringe needle inserted directly into the ventricle through a hole in the shell. Thereafter each of the 22 parts of interest was dissected out of the animal and placed immediately into ice-cold acetone, a solvent successfully used to extract FMRFamide and its congeners (Lehman *et al.* 1984; Price *et al.* 1985; Price, 1986).

The tissues were homogenized for 5 min (Polytron Model PT 10), the homogenate was centrifuged (10 000 ***g***, 15 min), and the pellet was re-extracted with 80% acetone. The two acetone supernatants were pooled and evaporated to dryness. The dried material was taken up in 1 ml of distilled water. Ten microlitres was removed, diluted, and these solutions were assayed with a radioimmunoassay (RIA) for FMRFamide.

The immunoreactive peaks recovered after gel filtration were pooled, lyophilized, taken up in buffer and subjected to high-pressure liquid chromatography (HPLC) (Waters Associates Model 720 System, with a μ-Bondapak C-18 reverse-phase column). Two solvent systems were used to characterize the peptides: an isocratic system consisting of 80% butanol-saturated buffer (0·5 mol l^{-1} ammonium acetate, 0·1 mol l^{-1} acetic acid, pH 5·5); and a gradient system comprising methanol and 1·0% trifluoroacetic acid (from 30% to 50% methanol in 20 min) at pH 2·0. The buffers were pumped at a rate of 2 ml min^{-1}, and the u.v. absorbance was monitored at 254 nm.

Radioimmunoassay

The antiserum

The antiserum was prepared in 1980 as follows. Tyr-Gly-Gly-Phe-Met-Arg-Phe-NH_2 (YGGFMRFamide) was coupled to thyroglobulin with a water-soluble carbodiimide (using the method of Skowsky & Fisher, 1972). The reaction mixture was diluted to approximately 1 mg ml^{-1} and emulsified in an equal volume of Freund's complete adjuvant. Approximately 1 ml of the emulsion was injected into many intradermal sites on the back of each of six rabbits. A booster injection of the same concentration, emulsified in Freund's incomplete adjuvant, was given 4 weeks later. Further booster injections were given 2 weeks prior to bleeding. An antiserum (S-253) obtained from a single bleeding of one rabbit has been used in all of the experiments described in this report. The structure–activity relationships of the RIA using this antibody have been well studied (Price, 1983). The most general

requirement for binding is the minimal sequence Arg-Phe-NH_2; other known peptides, including SCP_B and Met-enkephalin, do not significantly cross-react in the RIA (Fig. 1).

The tracer

YGGFMRFamide was iodinated monthly by the chloramine T method (Greenwood, Hunter & Glover, 1963). Chloramine T (10 μg) was added to ^{125}I (1·5 mCi) and YGGFMRFamide (1·0 μg) in 10·0 μl of sodium phosphate buffer, pH 7·6. After 15 s, 40 μg of sodium metabisulphate was added, and the iodinated YGGFFMRFamide was separated from free iodide with a Sep-Pak C-18 cartridge.

The assay

Each assay tube contained 10 μl of diluted antiserum (1:30 000), 50 μl of diluted trace (7000 counts min^{-1}) and 50 μl of either RIA buffer [1 % bovine serum albumin in phosphate-buffered saline (PBS), 0·01 % merthiolate, 0·025 mol l^{-1} sodium-EDTA] or sample diluted in the RIA buffer. The tubes were incubated at 4 °C overnight, and 1 ml of a charcoal dextran solution (0·01 mol l^{-1} sodium phosphate, pH 7·6; 0·01 % merthiolate; 2·5 g l^{-1} charcoal; and 0·25 g l^{-1} dextran) was added, and centrifuged (4000 rev. min^{-1}, 10 min, 4 °C) to separate the bound tracer from free tracer. Each sample was counted for 1 min and the percentage bound for unknowns was compared to an FMRFamide standard curve.

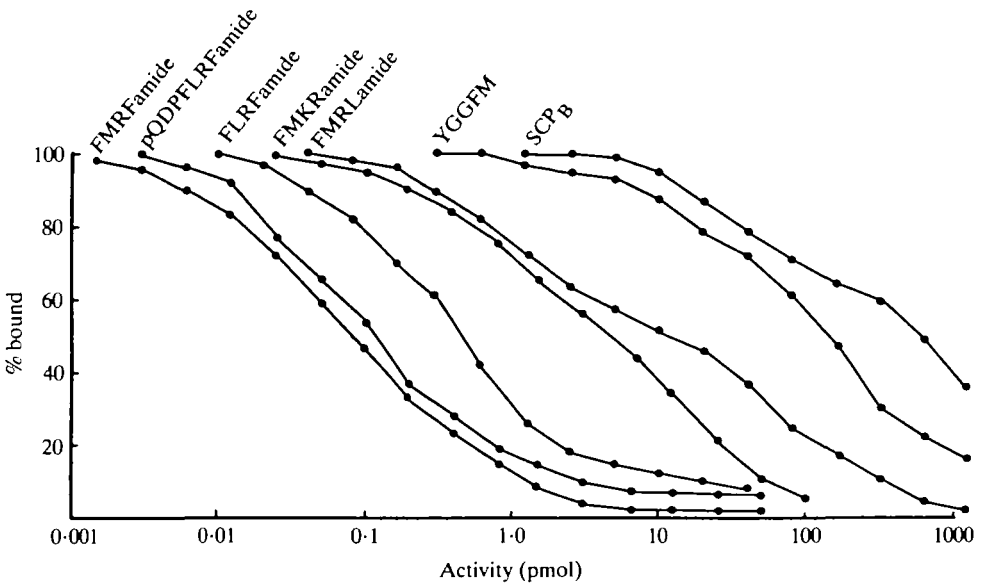

Fig. 1. Inhibition of binding of [^{125}I]YGGFMRFamide to antiserum S-253 by varying concentrations of FMRFamide, four structural analogues of FMRFamide, Met-enkephalin (YGGFM) and SCP_B. Details of the radioimmunoassay are given in the text.

Immunocytochemistry

The various tissues of *Helix aspersa* were fixed for 4 h with 4% paraformaldehyde in *Helix* saline (pH 7·6; Kerkut & Meech, 1965) and washed overnight with 30% sucrose in distilled water.

The fixed tissues were embedded in a gelatin–albumin mixture (35 ml of 6% gelatin, 15 ml of egg albumin) and frozen in liquid nitrogen. Sections (8–10 μm thick) were cut on a cryostat, mounted on slides coated with Histostik (Accurate Biochemicals), and frozen overnight. On the following day, the sections were incubated for 30 min with FMRFamide antiserum (S-253, diluted 1/200 in PBS), washed for 15 min with PBS, incubated for 30 min with fluorescein-labelled, goat anti-rabbit IgG (Boehringer Mannheim, diluted 1/50 in PBS and washed for 30 min with PBS). Two controls for specificity were applied: the antiserum was preincubated with FMRFamide, pQDPFLRFamide or SCP_B; and selected sections, prepared as described, were stained with non-immune serum.

The distribution of immunofluorescence was examined with a fluorescence microscope (Leitz Dialux 20) with incident light passed through a 436 nm excitation filter and a 490 nm suppression filter. All photographs were taken on Kodak Ektachrome 400 ASA film with a photoautomatic system (Wild MPS 50).

Bioassay

Radula protractor muscles, isolated from the whelk *Busycon contrarium* and prepared by the method of Hill (1958), were suspended in an organ bath containing aerated, natural sea water. Tension was recorded with a force-displacement transducer connected to an inkwriting oscillograph (Grass Instrument Co.). All drugs were added directly to the bath, and doses of FMRFamide are expressed in terms of their final concentrations in the bath (in $mol\,l^{-1}$).

RESULTS

Distribution and immunocytochemical localization

The concentrations of immunoreactive(ir-)FMRFamide in acetone extracts of various tissues were determined (Table 1).

Nervous system

In the central nervous system (CNS), ir-FMRFamide was concentrated in the suboesophageal and cerebral ganglia, and lesser quantities were found in the buccal ganglia. In addition to the material in the CNS, very large amounts of activity were found in the viscera.

Reproductive system

In the reproductive system, especially high levels of the peptides occurred in the male reproductive tract. The muscular epiphallus/flagellum complex contained levels exceeding even those in the ganglia, but the penis also possessed large

Table 1. *Distribution of ir-FMRFamide in the tissues of* Helix aspersa

System Tissue	*N*	Mass* (mg)	ir-FMRFamide† (nmol g^{-1} wet mass)
Nervous			
Suboesophageal ganglia	6	8·22	33·69 ± 27·50
Cerebral ganglia	6	3·95	43·17 ± 38·30
Buccal ganglia	1	1·32	1·80
Reproductive			
Epiphallus/Flagellum	4	14·49	68·15 ± 50·43
Penis	5	19·07	26·86 ± 26·94
Ovo-testis	3	40·69	3·18 ± 1·87
Common duct	4	79·94	1·85 ± 0·65
Spermatheca	2	7·53	1·76, 1·13
Mucus gland	2	8·10	0·98, 0·73
Dart sac	3	59·51	0·54 ± 0·06
Albumin gland	2	17·04	0·92, 0·05
Digestive			
Rectum	4	23·91	10·17 ± 9·14
Intestine	4	14·92	5·34 ± 3·42
Salivary gland	4	15·55	3·86 ± 3·45
Crop	5	40·60	2·07 ± 1·47
Buccal mass	3	49·70	1·08 ± 0·59
Digestive gland	2	88·85	0·24, 0·08
Circulatory			
Auricle	4	1·59	6·49 ± 3·72
Ventricle	4	4·02	2·46 ± 1·09
Blood	3		0·05 ± 0·02
Other			
Tentacle retractor muscle	5	10·49	24·69 ± 14·06
Collar	3	84·98	17·37 ± 18·78
Kidney	3	71·41	5·72 ± 4·07
Pharyngeal retractor muscle	3	7·82	0·88 ± 0·62

N, number of extracts assayed.
* Mean wet mass of individual tissues.
† nmol g^{-1} wet mass ± standard deviation, except where $N \leq 2$, then all values are given.
Acetone extracts of each tissue were prepared and assayed (see Materials and Methods).

amounts. The common duct, with both male and female components, had modest amounts of ir-FMRFamide, whereas other, particularly female, regions of the reproductive system had relatively low amounts (Table 1).

The distribution of immunocytochemically stained FMRFamidergic fibres in the reproductive system was also not uniform; i.e. the fibres were very dense in the male reproductive tract, but were rarely found in the female system. In the male portions, immunofluorescent fibres and varicosities were particularly localized in both the circular and longitudinal muscle layers in the thick walls of the penis, epiphallus, flagellum and dart sac (Fig. 2A). Only thin, sparsely distributed immunoreactive fibres were found in the vas deferens, and they were limited to the thin muscular sheath. Densely staining aggregates resembling cell bodies occurred between the muscle fibres in the outermost regions of the male tract.

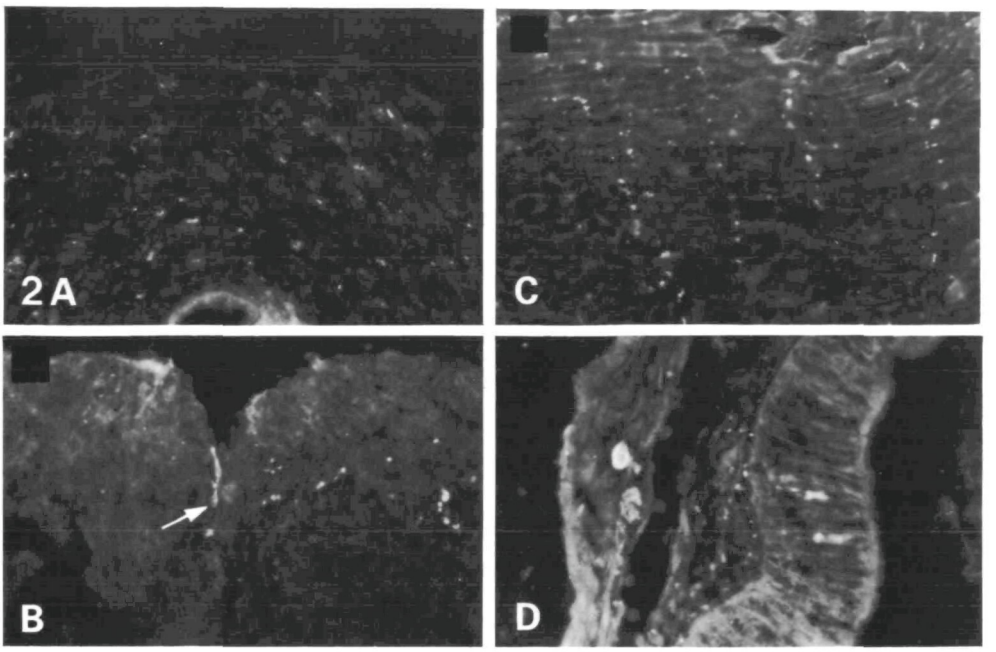

Fig. 2. Immunofluorescent photomicrographs of sections from the reproductive, digestive and muscular systems of *Helix aspersa* treated with FMRFamide-reactive antiserum S-253. (A) Immunofluorescent fibres in a cross-section through the epiphallus (×300). (B) Transverse section through the auriculoventricular junction (arrow) showing immunofluorescent FMRFamidergic fibres. The auricle is to the right (×300). (C) Longitudinal sections of the tentacle retractor muscle. Note the immunofluorescent FMRFamidergic fibres arranged in parallel rows (×300). (D) Section through the wall of the crop showing immunofluorescent cell bodies in the musculature underlying the serosal epithelium. Such positive perikarya were commonly observed in the digestive system (×300).

Digestive system

Within the digestive system, the rectum had the highest concentration of ir-FMRFamide, whereas more anterior regions of the tract contained less of the immunoreactive material. Significant levels were also found in the salivary glands (Table 1). The highest density of ir-FMRFamidergic nerves in the digestive system was found in the muscular layers of the rectum. Immunoreactive varicosities occurred in the circular muscle layer, and fibres extended into the muscular villi. No specific immunofluorescence was associated with the cells of the secretory mucosal epithelium of the rectum.

In all other regions of the digestive tract, including the intestine, crop and oesophagus, ir-FMRFamidergic fibres and varicosities were seen in the thin muscular sheath along the entire length of the digestive tract (Fig. 2D). As for the salivary glands, the muscular sheath surrounding the exocrine cells was densely stained.

Heart

The auricle contained most of the ir-FMRFamide in the heart; less was detected in the ventricle (Table 1). Immunoreactive FMRFamidergic fibres were scattered throughout the auricle in the outermost longitudinal muscle cell layer and the internal circular muscular layers. The ventricle contained relatively few immunoreactive fibres, but some were occasionally found at the auriculoventricular junction (Fig. 2B).

The concentration of ir-FMRFamide in the blood was determined from three pooled samples from 40 individuals and ranged from 10^{-9} to 10^{-8} mol l^{-1}. These values are in good agreement with those of Price *et al.* (1985).

Muscular system

The tentacle and pharyngeal retractor muscles are both part of the columnellar muscle group (Jones, 1975), but the concentrations of ir-FMRFamide in these two muscles were very different (Table 1). The tentacle retractor muscle contained the highest concentration of FMRFamide, and the largest number of ir-FMRFamidergic nerve fibres of all the muscles examined in *Helix*. These fibres were arranged in parallel rows, perpendicular to the longitudinal muscle cells (Fig. 2C). No staining was observed in the optic nerve or retina of the tentacle. Fluorescent fibres were also detected in the buccal musculature, and in the muscles of the foot and body wall. The pharyngeal retractor muscle and penis retractor muscle lacked ir-FMRFamide fibres.

Other

Immunoreactive FMRFamidergic fibres were observed in the muscular epithelium surrounding the kidney, collar, ovo-testis and digestive gland.

Biochemical characterization

When an extract of any tissue of *Helix* was subjected to gel filtration on a Sephadex G-15 column, the FMRFamide-like immunoreactivity migrated as two distinct peaks (Fig. 3). The first peak (designated 'peak C'), eluted in 2·5 void volumes and corresponds to the peak C originally described by Frontali *et al.* (1967). The second immunoreactive peak was retained longer and eluted in approximately 5–6 void volumes, but much earlier than the peak D of Frontali *et al.* (1967).

Peak C was separated into two major peaks by passage through a C-18 HPLC column in either of the two solvent systems (Fig. 4). The earliest peak eluted quickly and coincidentally with FMRFamide. This early peak migrated as a doublet in the butanol solvent system (Fig. 4A), the minor component eluting slightly before the FMRFamide standard. The second component of peak C appeared much later; it co-eluted with pQDPFLRFamide and was indistinguishable from a pQDPFLRFamide standard added to the extract before chromatography (Fig. 5).

The two HPLC peaks recovered from peak C were both active on the radula protractor muscle of *Busycon* (Fig. 6). Moreover, when doses equiactive on the

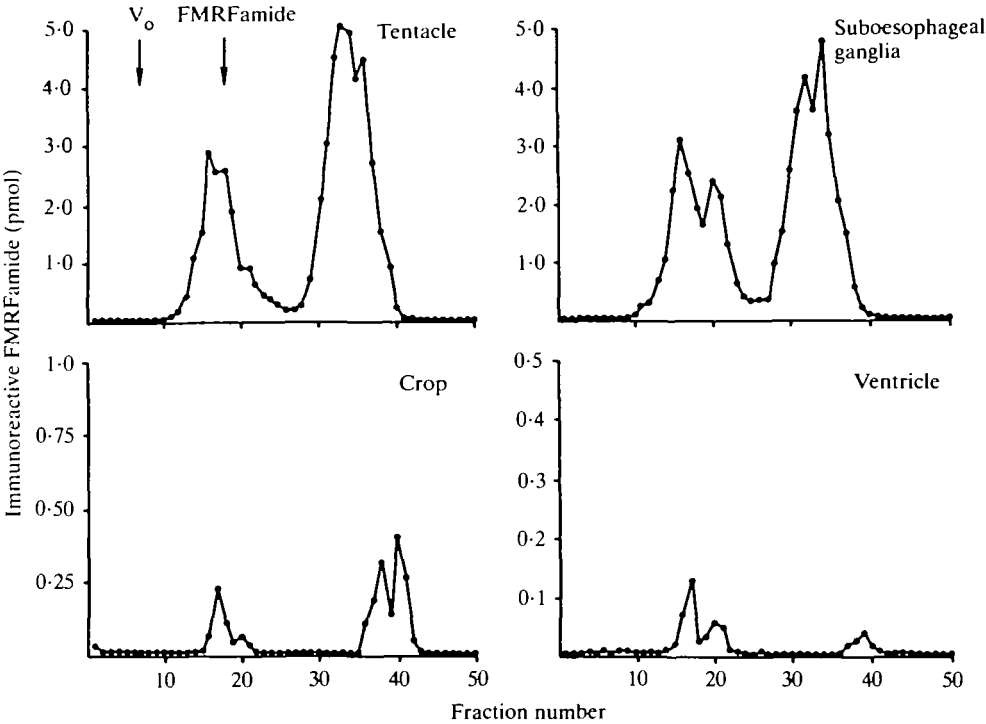

Fig. 3. Gel chromatography of tissue extracts from *Helix aspersa*. For each tissue an acetone extract of samples from 100 animals was passed through a 2·5 cm×43·5 cm Sephadex G-15 column previously equilibrated and eluted with 0·1 mol l^{-1} acetic acid. Ordinate: immunoreactivity measured in a 5 μl sample of each 8 ml fraction. Abscissa: fraction number. The elution position of the void volume (V_o) and of synthetic FMRFamide are indicated.

muscle were quantified by RIA, the peak eluting with FMRFamide contained 7–10 times less peptide (i.e. was more potent) than the peak eluting with pQDPFLRF-amide; the potencies of the synthetic peptides are of the same order on this assay system (Price *et al.* 1985).

FMRFamide and pQDPFLRFamide were not equally distributed in the tissues of *Helix* (Table 2). Whereas FMRFamide predominated in the reproductive system, retractor muscles and ventricle, pQDPFLRFamide was more abundant in the ganglia and digestive system.

The second peak eluting from the gel column (Fig. 3) migrated on HPLC as a single peak in both the isocratic and the gradient solvent systems; it eluted between FMRFamide and pQDPFLRFamide (Fig. 7). The material recovered from HPLC is immunoreactive, yet its immunoreactivity is not affected by treatment with carboxypeptidase Y, and its binding curve is steeper than that of either FMRFamide or peak C (Fig. 8). Moreover, 500 pmol of this HPLC peak, as determined by RIA, had no effect on the radula protractor muscle of *Busycon* which is sensitive to 4 pmol of authentic FMRFamide.

DISCUSSION

The ganglia, male reproductive organs and the posterior end of the digestive tract of *Helix* contained the highest levels of immunoreactive FMRFamide as determined by RIA, and these organs also had the highest density of immunocytochemically

Table 2. *Concentrations of FMRFamide-like and pQDPFLRFamide-like immunoreactivity in the tissues of* Helix aspersa

Tissue	FMRFamide	pQDPFLRFamide
Suboesophageal ganglia	5·07	10·33
Cerebral ganglia	1·28	12·95
Penis	12·36	1·82
Common duct	0·45	0·17
Crop	0·04	0·53
Rectum	0·56	1·21
Tentacle retractor muscle	4·90	1·23
Pharyngeal retractor muscle	0·08	0·02
Ventricle	1·51	0·16
Auricle	1·77	1·88

Acetone extracts of 100 tissues were prepared and chromatographed on Sephadex G-15. Immunoreactive peak C from G-15 was further chromatographed on a C-18 reverse-phase column; the immunoreactive peaks co-eluting with synthetic FMRFamide and pQDPFLRFamide were quantified by RIA. The values are averaged from three separate RIA determinations, and presented in nmol g^{-1} wet mass of tissue, corrected for losses during HPLC.

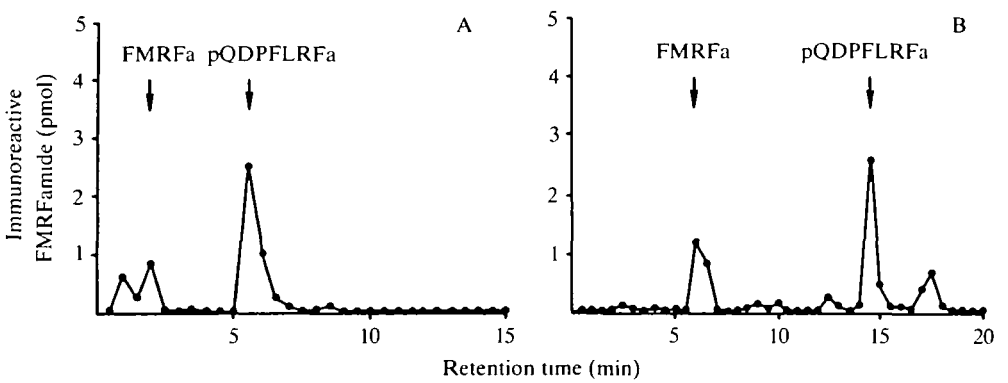

Fig. 4. Analysis on HPLC of peak C from suboesophageal ganglia of *Helix aspersa*. Peak C fractions (14–23) from a Sephadex G-15 column (see Fig. 4) were pooled, lyophilized and injected through a Waters μ-Bondapak C-18 column. (A) An isocratic elution profile employing 80% butanol-saturated buffer and 20% buffer pumped at 2 ml min^{-1}. Buffer: 0·5 mol l^{-1} ammonium acetate, 0·1 mol l^{-1} acetic acid. (B) A linear gradient profile from 30–50% methanol in 20 min (with 1% trifluoroacetic acid) pumped at 2 ml min^{-1}. Immunoreactivity was measured in a 5 μl sample of each 1·0 ml fraction. The retention times of synthetic FMRFamide (FMRFa) and pQDPFLRFamide (pQDPFLRFa) are indicated.

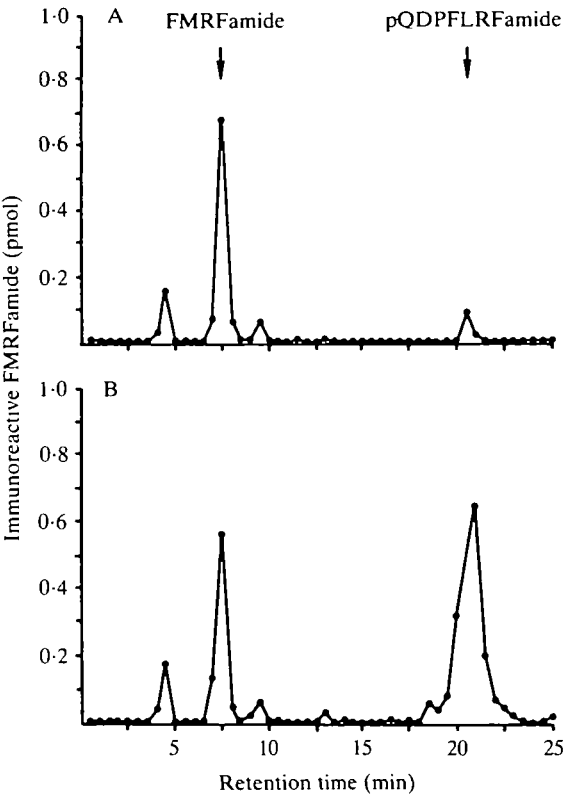

Fig. 5. Synthetic and natural pQDPFLRFamide co-eluted on HPLC. (A) Chromatogram of a sample of peak C, isolated from the penis and eluted with a methanol–trifluoroacetic acid gradient (see Fig. 4 legend). (B) Chromatogram of an equal sample of peak C to which 0·5 nmol of pQDPFLRFamide had been added. The retention times of synthetic FMRFamide and pQDPFLRFamide are indicated.

positive neural structures. In the non-neural organs, FMRFamide-like immunoreactivity was confined to muscle tissue and was observed mostly as nerve fibres, though a few nerve cell bodies were seen in the digestive and male reproductive tracts. These results are very similar to those of two previous immunocytochemical studies on *Helix aspersa*, one on the circumoesophageal ganglia (Marchand, Wijdenes & Schot, 1982) and the other on the peripheral nervous system (Cardot & Fellman, 1983). In particular, Cardot & Fellman found, as we did, that most of the immunoreactivity is contained in muscle tissue, with the digestive and male reproductive systems having especially dense innervations and even some reactive perikarya.

These findings show that muscle is the primary peripheral target of FMRFamidergic innervation. Other studies had indicated that these peptides, serving as transmitters or as modulators, are able to regulate the contractility of *Helix* muscles (Cottrell *et al.* 1983*a*,*b*; Lehman & Greenberg, 1987). Since we also found relatively high levels of FMRFamide-like immunoreactivity in the blood, some or all of these peptides might also be hormones. We presume that their actions are, again, on muscle, but we cannot rule out other targets.

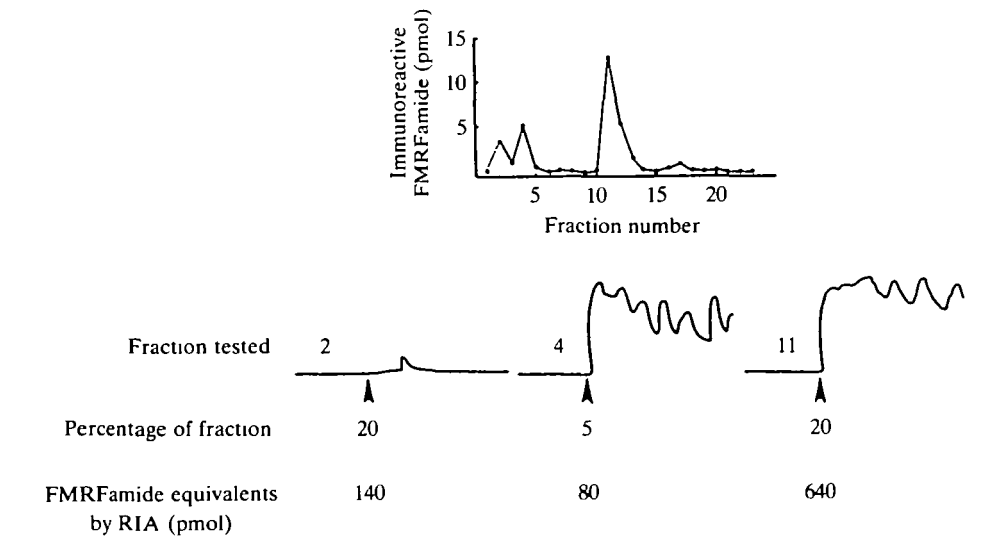

Fig. 6. Biological activity of peak C components. Peak C was purified from extracts of cerebral ganglia and chromatographed on HPLC with a butanol–trifluoroacetic acid gradient. Each fraction recovered from HPLC was quantified by RIA (top diagram). Samples of the immunoreactive peaks – 2, 4 and 11 – were tested on the radula protractor muscle of *Busycon* (mechanical records). Fraction 11 (corresponding to the elution of pQDPFLRFamide) contained most of the immunoreactive material. Fraction 4 (corresponding to FMRFamide) was most potent on the radula protractor. That is, 5% of fraction 4 (containing 80 pmol of immunoreactive FMRFamide) was equipotent with 20% of fraction 11 (containing 640 pmol of immunoreactive FMRFamide). Fraction 2 (corresponding to oxidized FMRFamide) was relatively inactive on the radula protractor muscle.

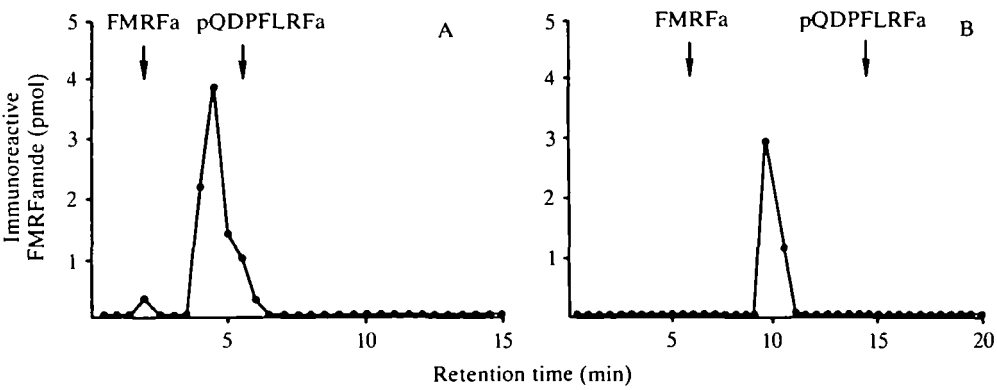

Fig. 7. Chromatography of the retained peak of immunoreactivity obtained by passing extracts of suboesophageal ganglia through Sephadex G-15 (fractions 30–35). The Sephadex fractions were pooled, lyophilized and analysed on an HPLC column. (A) An isocratic elution profile (80% butanol-saturated buffer and 20% buffer; pumped at 2 ml min^{-1}). (B) A linear gradient profile (30–50% methanol in 20 min, with 1% trifluoroacetic acid; pumped at 2 ml min^{-1}). Immunoreactivity was measured in a 5 μl sample of each 1·0 ml fraction. The retention times of synthetic FMRFamide (FMRFa) and pQDPFLRFamide (pQDPFLRFa) are indicated.

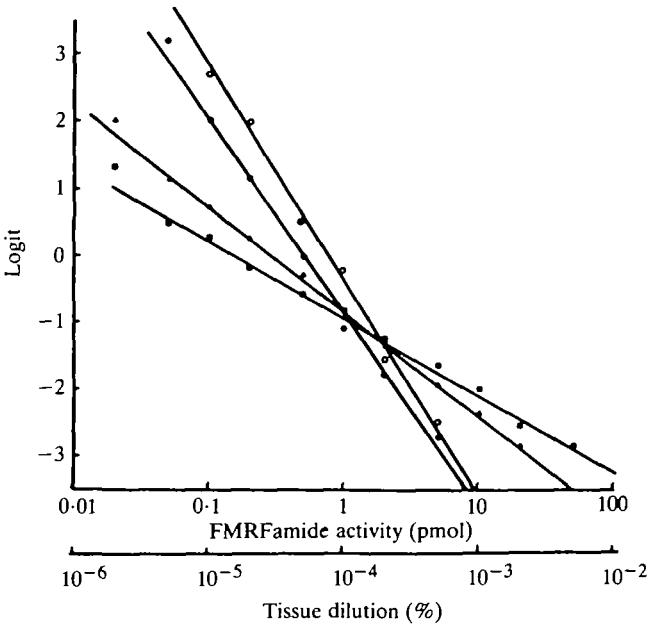

Fig. 8. Inhibition of binding of ^{125}I-YGGFMRFamide to antiserum S-253 by synthetic FMRFamide (squares) and the immunoreactive peaks obtained by passing suboesophageal ganglia extracts through Sephadex G-15. Triangles, peak C; closed circles, retained peak (fractions 30–35; see Fig. 8); open circles, retained peak digested with 10 μl of a 1 mg ml^{-1} solution of carboxypeptidase Y at 37°C overnight.

The FMRFamide-like immunoreactivity extracted from *Helix* is composed of several chromatographically distinct substances. On Sephadex G-15, two large, broad peaks of immunoreactivity are separated. The earlier, eluting at 2·5–3·0 void volumes, can be identified with the peak C defined by Frontali *et al.* (1967). Peak C is found in all molluscan species and several FMRFamide-like peptides have been isolated from it, including FMRFamide, FLRFamide and pQDPFLRFamide (Price *et al.* 1985; Price, 1986). Elution at this position is characteristic of these peptides because of the interaction of their aromatic residues with the gel (Gelotte, 1960).

The second peak on Sephadex G-15 is strongly retarded by the gel; its elution position is somewhat variable (Fig. 3) – about two column volumes (5–6 void volumes) – and it has virtually no mechanical activity on the radula protractor muscle. On HPLC, it elutes as a single peak of immunoreactivity between FMRFamide and pQDPFLRFamide, but its dilution curve in the RIA is steeper than that of FMRFamide or pQDPFLRFamide. Furthermore, it is insensitive to carboxypeptidase Y digestion. The exact nature of this non-specific immunoreactivity is still unknown, but it appears to have none of the biological or chemical features of FMRFamide. PseudoFMRFamide has been found in other molluscan species and has been detected with antisera other than our own (Price, 1986).

Although crude extracts contain pseudoFMRFamide, the data in Table 1 are still useful because the levels of pseudoFMRFamide present in the tissues are roughly

proportional to the level of authentic peptide. This relationship is evident in Fig. 3 which shows that where the authentic peptide peak is large (tentacle and suboesophageal ganglion), the pseudoFMRFamide peak is also large, and where the peptide peak is small (crop and heart), the pseudoFMRFamide peak is also small. It is this proportionality which allowed us to use the immunoreactivity of the acetone extracts as a crude measure of the amount of FMRFamide-like peptide contained in the tissues of the snail.

When peak C (i.e. authentic peptide) is chromatographed on a reverse-phase column, two major peaks of immunoreactivity can be identified with known peptides. The first of these is FMRFamide and the second is pQDPFLRFamide. FMRFamide had previously been reported to be absent from *Helix*, but the work presented here clearly shows that this conclusion was wrong; it also provides an explanation for the erroneous conclusion, as follows.

The absence of FMRFamide was first suggested by Cottrell, Price & Greenberg (1981). Using an extensive extraction and purification protocol, and a bioassay to detect activity, these authors reported that, unlike authentic FMRFamide, the majority of FMRFamide-like activity was not retained by a cation exchange column. Later Price (1982), equipped with a more efficient extraction technique, better purification methods (HPLC) and a more sensitive assay (radioimmunoassay), detected several peaks of FMRFamide-like activity but focused on the sequence of the major peptide, which was finally shown to be pQDPFLRFamide (Price *et al.* 1985). In the earlier study the extracts were handled extensively, increasing the likelihood that FMRFamide would be oxidized. In this investigation, we handled the material minimally, but still observed a peak on HPLC which preceded that of FMRFamide and co-eluted with oxidized, synthetic FMRFamide. Moreover, this peak had very little biological activity compared with its immunoreactivity (Fig. 6). Therefore, if the majority of FMRFamide in an extract were to be oxidized, and only a bioassay were to be available, the pQDPFLRFamide would be the only peptide detected.

In addition to the major FMRFamide and pQDPFLRFamide peaks we also see some minor peaks on HPLC. One is probably an analogue of pQDPFLRFamide with a different *N*-terminal amino acid, and another may be FLRFamide (Price *et al.* 1985; Price, 1986). But these minor peaks occur at such low levels that we have not yet isolated enough material to make an unequivocal identification. FLRFamide not only occurs at a very low level, but it also has a low immunoreactivity with our antibody, which compounds the problem of detecting it.

The importance of the antiserum is illustrated by the results of Dockray *et al.* (1981), who also used an RIA to measure the level of FMRFamide-like immunoreactivity in the circumoesophageal ganglia of *Helix*. The concentration that they measured in the suboesophageal ganglia ($18{\cdot}2 \pm 6{\cdot}6\,\mathrm{nmol\,g^{-1}}$, ±S.E.) was very similar to our determinations: $33{\cdot}69 \pm 27{\cdot}5\,\mathrm{nmol\,g^{-1}}$ (±S.D.) (Table 1) and $15{\cdot}4\,\mathrm{nmol\,g^{-1}}$ (the sum of FMRFamide and pQDPFLRFamide in Table 2). The agreement is particularly surprising because the extraction procedures and the antisera were different, and the antisera even have radically different specificities

(Dockray, 1985). In the cerebral ganglia, however, Dockray *et al.* (1981) found only 4·2 nmol g^{-1} of FMRFamide immunoreactivity (about one-quarter of the level in the suboesophageal ganglia), whereas we found that the total immunoreactivity was about equal in the two tissues (Tables 1, 2). But we did find that the cerebral ganglia have only one-quarter as much FMRFamide as the suboesophageal ganglia, the balance in the cerebral ganglia being achieved by a higher level of pQDPFLRFamide (Table 2). The two sets of results are reconcilable in terms of known differences in the antisera. Our RIA reacts about equally with pQDPFLRFamide and FMRFamide (Fig. 1; Price *et al.* 1985; Price, 1986), whereas Dockray's antiserum should only cross-react 10 % as well with pQDPFLRFamide as with FMRFamide, based on its reactivity with other peptides having a leucine residue in place of the methionine (Dockray, 1985). Therefore, the measurements of Dockray *et al.* (1981) support the data reported here, and they are also consistent with our finding that the ratio of the levels of FMRFamide and pQDPFLRFamide vary from tissue to tissue (Figs 4A, 5A; Table 2).

The variation in the ratio of FMRFamide and pQDPFLRFamide might reflect differential processing of a single gene product (at either the RNA or the protein level) or the expression of distinct FMRFamide-encoding genes in different anatomical regions. Both mechanisms are known to account for neuropeptide diversity in *Aplysia* (Scheller *et al.* 1982; Schaefer *et al.* 1985). However, the variable ratio might only reflect differential recovery or degradation in the different regions. It is important to clone the genes involved to answer this question more definitively.

This variable ratio suggests that FMRFamide and pQDPFLRFamide might serve different functions, a notion supported by the differential actions of the two peptides on *Helix* muscle (Lehman & Greenberg, 1987). Another possibility is that one of these peptides is delivered preferentially to the tissues *via* the blood. Since pQDPFLRFamide has a blocked *N*-terminal, it is partially protected from enzymatic degradation and, therefore, might serve as a hormone. Moreover, pQDPFLRFamide does occur at pharmacologically active levels in the blood of *Helix* (Price *et al.* 1985).

Several workers have examined the actions of FMRFamide on molluscan neurones (Cottrell, Davies & Green, 1984; Stone & Mayeri, 1981), gut (Austin *et al.* 1983; Doble & Greenberg, 1982) and retractor muscles (Cottrell *et al.* 1983*a*; Painter, 1982; Nagle & Greenberg, 1982) and all have suggested that FMRFamide is a neurotransmitter or modulator in these tissues. This study suggests that in addition to their action in those tissues, FMRFamide and pQDPFLRFamide may act as a transmitter or modulator in the mediation of muscular movements of the male reproductive tract. In other molluscs, *Aplysia* and *Lymnaea*, immunoreactive FMRFamide is highly concentrated in the male reproductive organs (H. K. Lehman, unpublished observations). Thus, in pulmonates and opisthobranchs, the FMRFamide family of peptides may be important regulators of reproduction, as well as of other central and visceral functions.

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