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CARDIAC REGULATION BY ENDOGENOUS SMALL CARDIOACTIVE PEPTIDES AND FMRFamide-RELATED PEPTIDES IN THE SNAIL *HELIX ASPERSA*

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Summary

The putative heptapeptide pQDPFLRIamide, previously known only from its appearance in a cDNA clone from *Helix aspersa*, was isolated from circumoesophageal ganglia extracts and sequenced.

Extracts of several tissues were fractionated by high performance liquid chromatography and the fractions analysed by radioimmunoassay (RIA). The results indicate that ten cardioactive peptides, FMRFamide, FLRFamide, six FMRFamide-related heptapeptides and two nonapeptide analogues of the small cardioactive peptides (SCPs), are present in the circumoesophageal ganglia (brain), the visceral nerve trunk (from which the cardiac nerve branches) and the aorta. The heart contains the two tetrapeptides, FMRFamide and FLRFamide, and the SCPs, but the heptapeptides were completely undetectable in this organ. The levels of tetrapeptide were high enough to allow their calcium-dependent release from the heart to be demonstrated. Immunohistochemistry revealed a diffuse SCP and FMRFamidergic innervation distributed throughout the heart. These data support the idea that, although the ten peptides are probably acting as neuro*transmitters* throughout most of the cardiovascular system, the heptapeptides probably also have a neuro*hormonal* rôle on the *Helix aspersa* heart itself. The binding affinities of the various antisera used in these studies were examined in competitive RIAs, in non-competitive dot-blot assays or in both.

Introduction

Nine cardioactive peptides have been isolated from the pulmonate snail *Helix aspersa*: two SCPs (small cardioactive peptides) and seven analogues of FMRFamide (Price *et al.* 1990). The FMRFamide analogues consist of two tetrapeptides and five heptapeptides.

Two series of cDNA clones have also been sequenced from *H. aspersa* (Lutz *et al.* 1990). One encodes only the two tetrapeptides FMRFamide and FLRFamide, whereas the other encodes the sequences for the five known heptapeptides plus three additional putative peptides. One of these putative peptides, pQDPFLRIamide, is clearly homologous to the other heptapeptides, but is unique in that it ends with -RIamide rather than -RFamide. As the tetrapeptides and heptapeptides are encoded in separate transcripts, we reasoned that an antiserum that would bind to pQDPFLRIamide could be

Key words: FMRFamide, SCP, mollusc, neuropeptide, heart, snail, *Helix aspersa*, biocytin.

FMRFamides

Phe-Met-Arg-Phe-NH_2	FMRFamide
Phe-Leu-Arg-Phe-NH_2	FLRFamide
Asn-Asp-Pro-Phe-Leu-Arg-Phe-NH_2	NDPFLRFamide
Asn-Asp-Pro-Tyr-Leu-Arg-Phe-NH_2	NDPYLRFamide
Ser-Asp-Pro-Phe-Leu-Arg-Phe-NH_2	SDPFLRFamide
Ser-Glu-Pro-Tyr-Leu-Arg-Phe-NH_2	SEPYLRFamide
Glp-Asp-Pro-Phe-Leu-Arg-Phe-NH_2	pQDPFLRFamide
Glp-Asp-Pro-Phe-Leu-Arg-Ile-NH_2	pQDPFLRIamide

SCPs

Met-Asn-Tyr-Leu-Ala-Phe-Pro-Arg-Met-NH_2	MNYLAFPRMamide
Ser-Gly-Tyr-Leu-Ala-Phe-Pro-Arg-Met-NH_2	SGYLAFPRMamide

Fig. 1. The endogenous cardioactive peptides of *Helix aspersa*. Both the three-letter and one-letter abbreviations for the amino acids are given.

used to mark the expression of the heptapeptides, provided this peptide is actually processed. In this report we describe the isolation and identification of pQDPFLRIamide from extracts of *H. aspersa* ganglia and its cardioactivity. The sequences of all ten identified cardioactive peptides of *Helix aspersa* are listed in Fig. 1.

Although the cardioactivities of these ten peptides have been confirmed in *H. aspersa*, their sites of synthesis, modes of delivery to their target organs, mechanisms of action and physiological roles, singly and in combination, remain obscure.

In this paper, the differential distributions of the ten peptides in the cardiovascular system, investigated with high performance liquid chromatography (HPLC) and radioimmunoassay (RIA) of tissue extracts and by immunohistochemistry are described. Additionally, the calcium-dependent release of the FMRFamide analogues from the heart is demonstrated as a first step towards elucidating the rôles of these peptides in the regulation of cardiac physiology in *Helix aspersa.*

Materials and methods

Helix aspersa Müller were collected and shipped from Fullerton, California, to St Augustine, Florida, by Dr R. Koch. As in earlier experiments (Price *et al.* 1990), the animals were allowed to remain dormant for several months before being used for bioassay or for quantitative analysis of the peptide distributions; they survived better in this state. Other snails were moved to moist chambers a few days before they were used for immunohistochemical or nerve tracing studies as preliminary experiments had revealed no obvious difference in the immunohistochemical staining patterns of cardiovascular tissues from active and dormant animals (W. Lesser, unpublished data).

Some peptides were purchased from Peninsula Laboratories Inc., Cambridge Research Biochemicals or Sigma; others were synthesised by the Protein Sequence Core Facility of

Table 1. *Primary antisera, antigens and uses*

Antiserum	Antigen	Protocol
S253	YGGFMRFamide	Radioimmunoassay
Q2	pQDPFLRFamide DDPFLRFamide	Radioimmunoassay
Polyclonal SCP_B	MNYLAFPRMamide	Radioimmunoassay
EFLRIamide	EFLRIamide	Radioimmunoassay Immunohistochemistry
Weber	FMRFamide	Immunohistochemistry
Monoclonal SCP_B	MNYLAFPRMamide	Immunohistochemistry

A list of the antisera used, the antigens against which they were raised, and the protocols in which they were used.

All the antisera were rabbit polyclonals except the SCP_B mouse monoclonal.

Conjugation details and sources are provided in the text.

the University of Florida Interdisciplinary Center for Biotechnology Research (SGYLAFPRMamide, NDPFLRFamide, NDPYLRFamide, SEPYLRFamide, pQDPFLRIamide and YPFLRIamide). The synthetic peptides were deprotected and purified by K. E. Doble (this laboratory).

The S253 antiserum was raised against a conjugate of YGGFMRFamide and the Q2 antiserum against pQDPFLRFamide and DDPFLRFamide (Price *et al.* 1990). The antiserum against EFLRIamide, which is a peptide predicted from a gene sequence obtained from *Lymnaea stagnalis* (Linacre *et al.* 1990), was raised in rabbit to an EFLRIamide–bovine thyroglobulin conjugate (Santama, 1992). A polyclonal antiserum to SCP_B (MNYLAFPRMamide) was provided by Dr H. Morris (Imperial College, London), and a monoclonal antibody (Masinovsky *et al.* 1988) by Dr A. O. D. Willows (Friday Harbor Laboratory, University of Washington). The polyclonal antiserum was used in the RIAs while the monoclonal antiserum was reserved for immunohistochemistry. Dr E. Weber (University of California) provided an antiserum raised against FMRFamide (Weber *et al.* 1981). The primary antisera names, antigens and uses are summarised in Table 1. Normal goat serum was purchased from Boehringer Mannheim Biochemicals and the secondary and tertiary antisera from Sigma. The remaining chemicals and supplies were purchased from Sigma or Fisher.

All experiments were repeated at least three times; representative results from a single experiment are presented where it is not practical to show pooled data.

The anatomy of the snail

A dissected animal is illustrated in Fig. 2. The animal was anaesthetised with $MgCl_2$; the shell was removed and incisions made as indicated (Fig. 2A). The lung was displaced to the left and the reproductive organs to the right. The remaining tissues were displaced as little as possible; moreover, apart from trimming the peritoneal wall and severing the penis retractor muscle, no other cuts were made. Much of the digestive tract and liver are obscured by overlying tissue. The vessels and nerve trunks visible under the dissecting

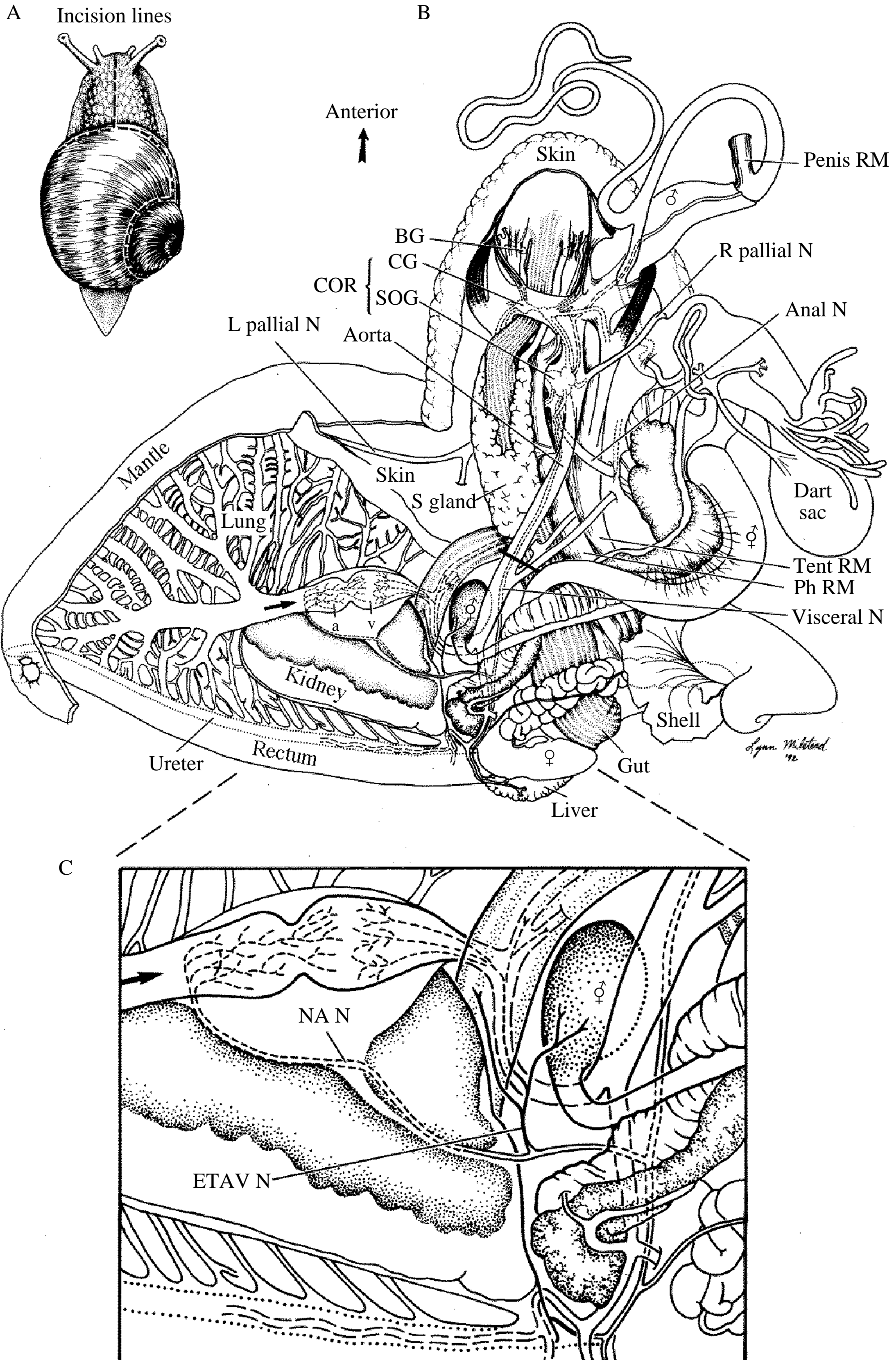

Fig. 2

Fig. 2. Drawing of a dissected *Helix aspersa*. (A) The shell was removed and the indicated incisions were made (heavy broken lines). (B) The complete animal. The lung was displaced to the left, the reproductive organs to the right [they would normally lie over the aorta between the brain and the albumen gland (♀) in the intact animal] and the penis retractor muscle has been severed. 'Skin' includes the peritoneum (separating the main body cavity from the lung in the snail); this has been trimmed, especially on the left side, to expose the lung. Broken lines outline organs lying beneath other tissues; the nervous tissue is outlined by short broken lines, the circulation is outlined by long broken lines and other organs are outlined by dotted lines. The division between the proximal and distal segments of the aorta is indicated by the heavy solid line. Much of the gut and liver are obscured by overlying tissue, though a partial liver lobe is visible. With the exception of the cardiac innervation, only structures visible with the naked eye or dissecting microscope have been drawn, so that some nerves or vessels appear to end abruptly. (C) The cardiac region and branching of the visceral nerve. Note that, as the visceral nerve approaches the heart, it branches frequently to innervate the gonads, liver, gut, rectum and ureter as well as the aorta, heart and, probably, the kidney. The sexually mature animal is 3–5cm from head to foot. BG, buccal ganglia; CG, cerebral ganglia; SOG, suboesophageal ganglia; COR, circumoesophageal ring; L pallial N, left pallial nerve; R pallial N, right pallial nerve; Anal N, anal nerve; Visceral N, visceral nerve; NA N, nephroatrial nerve; ETAV N, entero-theco-arterio-ventricular nerve; Penis RM, penis retractor muscle; Tent RM, tentacle retractor muscle; Ph RM, pharyngeal retractor muscle; S gland, salivary glands; a, atrium; v, ventricle; sex or hermaphroditic symbols indicate various reproductive organs.

microscope are indicated. The innervation of the heart, elucidated in the Results of this report, is also shown.

Isolation and purification of pQDPFLRIamide

The circumoesophageal rings (total wet mass 0.8g) were dissected from 50 animals and immersed in 3.2ml of acetone overnight at −20˚C. The acetone extract was decanted from the tissue, which was then rinsed in several more volumes of acetone, and the extract and rinse were pooled. The acetone was evaporated with an air stream. Several volumes of aqueous 0.1% trifluoroacetic acid (TFA) were added to the residue, and the resulting solution was loaded onto a reverse-phase Brownlee RP-300 (2.1mm×220mm) HPLC column at 0.5mlmin^{-1} as described by Price *et al.* (1990).

Four HPLC runs, listed and described in detail below, sufficed to purify the peptide for fast atom bombardment mass spectroscopy–mass spectroscopical (FABms-ms) analysis. The four-step HPLC purification proceeded as follows: (1) a linear gradient of 8.0% to 40.0% acetonitrile (ACN) in 0.1% TFA over 40min; (2) a linear gradient of 8.0% to 40.0% ACN in 0.05% heptafluorobutyric acid over 40min; (3) a gradient composed of three linear segments of ACN in 5mmoll^{-1} phosphate buffer, pH7.0: (a) 0% to 6% over 5 min, (b) 6.0% to 48.0% over 15min and (c) 48.0% to 60.0% over 10min; and (4) a linear gradient of 8.0% to 40.0% ACN over 40min with 0.1% TFA. Fractions were collected for 30s, and a 1 μl sample was taken from each fraction for RIA. The one or two most immunoreactive fractions from each HPLC run were pooled and injected onto the column for the subsequent gradient. Fraction 47 from the final run was dried in a SpeedVac concentrator (Savant) and analysed by FABms-ms. Fraction 46 was saved and applied to the column with synthetic pQDPFLRIamide.

Characteristics of the antisera in an RIA

The binding affinities and specificities of the four polyclonal antisera, S253, Q2, EFLRIamide and SCP_B, used in the RIAs were investigated. The ten identified endogenous peptides, as well as seven putative peptides and analogues, were incubated with the antisera (using the protocol of Price *et al.* 1987). The resulting binding curves were fitted by computer; i.e. the method of least squares was applied to the data points after log/logit transformation. The IC_{50} values from several runs were averaged.

Characteristics of the antisera in a dot-blot assay

Most of the known cardioactive peptides, as well as two synthetic peptides, were covalently linked at their N terminals to bovine serum albumin (BSA) (Tager, 1976). The N-terminally protected peptides, pQDPFLRFamide and pQDPFLRIamide, cannot be linked in this manner and were, therefore, represented by NDPFLRFamide and YPFLRIamide, respectively. The extent of conjugation to BSA cannot readily be determined and it was variable. Two peptides (ENNNGYIRFamide and MNYLAFPRMamide) conjugated very poorly and were therefore not tested in these experiments.

The conjugates were serially diluted twofold, and each dilution was dotted onto 0.45 μm nitrocellulose membranes. After fixation in 5:1:5 methanol:acetic acid:water, the membranes were incubated in one of three primary antisera (Weber's, EFLRIamide and monoclonal SCP_B); the same dilutions that were used to stain the tissues were used in the dot-blot assay. This incubation was followed by treatment with ^{125}I-labelled secondary antibodies (Jahn *et al.* 1984). The binding was visualised with autoradiographs exposed overnight.

Preparation of extracts and analysis by HPLC and RIA

Tissue pooled from as few as five animals provided sufficient material for a quantitative analysis, though an extract of 100 hearts was also analysed to confirm the absence of the FMRFamide-like heptapeptides in this organ. The tissues within the pericardial cavity, the atrium and ventricle, were removed from the snail and analysed together as heart tissue. The aorta was analysed in two segments: the proximal (from the heart to, and including, the stumps of the branches supplying the gut and body wall) and the distal (continuing on to the suboesophageal ganglia). These two segments are indicated in Fig. 2B.

Two types of nervous tissue were assayed: the circumoesophageal ring (brain) and the visceral nerve trunk. Only the portion of the visceral nerve trunk running adjacent to the distal segment of the aorta is free of other organs and, to minimise contamination, only this segment (about half the nerve) was used.

Once excised from the snail, the tissues were weighed, and each type was immersed in four times its mass of acetone and stored at −20˚C overnight. The aortas were flushed with saline before their immersion in acetone to remove any haemolymph within them.

After 12–18h of incubation, the acetone extract was decanted, concentrated and fractionated by HPLC in a linear gradient of 8.0% to 40.0% ACN in 0.1% TFA over

40min. Fractions were collected for 30s. Samples (1–5 μl) were taken from fractions 21–60 (renumbered 1–40 in Figs 8–12) for RIA with each of the four polyclonal antisera. In some instances, the fractions were dried in a SpeedVac concentrator and the residue was dissolved in the RIA buffer; larger samples could thus be assayed.

In addition to the solid tissues, haemolymph was collected from the vena magna (Renwrantz *et al.* 1981) of 8–10 aestivating animals and pooled to yield 4ml. The haemolymph was injected into 16ml of acetone on ice as it was collected. The mixture was centrifuged briefly and stored at −20˚C overnight. The following day, the extract was swirled, recentrifuged, the supernatant decanted and the acetone was evaporated under vacuum at 65˚C. The aqueous residue was centrifuged, and the supernatant was loaded onto the column, fractionated and assayed as above. In one experiment, 4pmol of synthetic pQDPFLRIamide (equivalent to 1nmol l^{-1} in the 4ml collected) was added to the haemolymph and an equal amount of synthetic pQDPFLRIamide was added to 4ml of saline. Both volumes were extracted in acetone, fractionated and assayed as previously described.

Calcium-dependent release of the FMRFamide peptides

The heart was cannulated at the aortic end of the ventricle. The aorta was cut distal to the insertion of the cannula, and the atrium was cut at its junction with the pulmonary vein. The cannula was connected to a loop system, devised by Payza (1987), but modified as follows. The dead space in the loop was minimised by connecting the three-way taps directly to the Y-tubes, especially at the sample loop, so that a sharp front of saline would enter the heart (Fig. 3). The sample loop had a volume of 1.8ml. The perfusion rate was set very low (20 μl min^{-1}).

The heart was first perfused with a normal saline (7mmol l^{-1} $CaCl_2$, 4mmol l^{-1} KCl, 5 mmol l^{-1} $MgCl_2$, 80mmol l^{-1} NaCl, 5mmol l^{-1} Tris–HCl, pH7.5) for 30min to allow for equilibration and to wash away the haemolymph and its active components. Drops of perfusate were then collected in sequence, one drop per tube. After five drops had been collected, the sample loop was filled with 0mmol l^{-1} $CaCl_2$/40mmol l^{-1} KCl saline (calcium was replaced by magnesium, and sodium by potassium, maintaining the monovalent and divalent ion concentrations), and 30 more drops were collected. The saline was then returned to its starting composition, and a further 30 drops collected. Finally, the heart was perfused with 7mmol l^{-1} $CaCl_2$/40mmol l^{-1} KCl saline (i.e. normal calcium, high potassium) and 30 drops were collected.

At the end of the experiment, each tube was assayed by RIA, with each 40 μl drop serving as a sample. The values for the successive tubes were subjected to a running average of three; this reduced the effect of stray drops and clarified the overall pattern of release.

Visualisation of the cardiac innervation with biocytin

In the first experiment, the freshly cut end of the visceral nerve trunk was incubated in a pool of biocytin (Molecular Probes, 4% in 0.05mol l^{-1} Tris/0.5mol l^{-1} KCl, pH7.5) for 4–7 days, during which time the biocytin was transported to the heart. After the

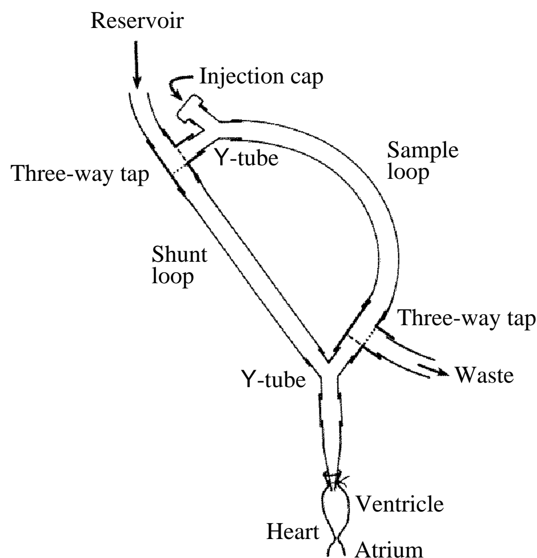

Fig. 3. The closed-loop system for the stimulated release of FMRFamide peptides from the isolated heart (modified from Payza, 1987). When the taps are adjusted to prevent saline flowing across the broken (dashed) lines in the three-way taps, the saline flows through the shunt loop and the desired saline can be injected into the sample loop through the injection cap, displacing the saline already present, which flows out through the waste tubing. If the taps are then adjusted so that flow across the dotted lines is obstructed, the contents of the sample loop will flow through the heart. Note that the flow through the heart is from the ventricle to the atrium. This allowed the atrium, ventricle and atrial-ventricular junction all to be exposed to the salines and thus to contribute to the release of peptides.

incubation, the heart was excised and fixed overnight in 3% paraformaldehyde in 0.1 mol l^{-1} phosphate buffer. The heart was rinsed in several changes of phosphate buffer over 2h, then incubated in an avidin:biotinylated horseradish peroxidase complex (ABC solution, Vector Labs), rinsed, incubated in 0.5% 3,3′-diaminobenzidine (DAB) (Sigma) for 20min, and developed in H_2O_2. The reaction was quenched in buffer, the tissues were mounted on glass slides, dehydrated in alcohols and xylene then mounted in Permount (Fisher) under coverslips. This method was adapted from Horikawa and Armstrong (1988). In subsequent preparations, all of the branches, except the one presumed to innervate either the atrium or the ventricle, were cut; thus, only the atrial or ventricular branch was filled in a given preparation. The tissue was fixed and processed as before.

Immunohistochemistry of the cardiovascular system

After overnight fixation in 3% paraformaldehyde, the tissue was rinsed, blocked in antibody diluent (AD: 0.3% Triton X-100, 5% normal goat serum, 3% bovine serum albumin in 0.1 mol l^{-1} phosphate buffer), then incubated overnight in the primary antibody (Weber 1:1000, EFLRIamide 1:1000, monoclonal SCP_B 1:50). After rinsing, the tissue was incubated in the secondary antibody (goat-anti-rabbit or goat-anti-mouse in AD, both at 1:100), rinsed again, incubated in rabbit-peroxidase anti-peroxidase (PAP) or

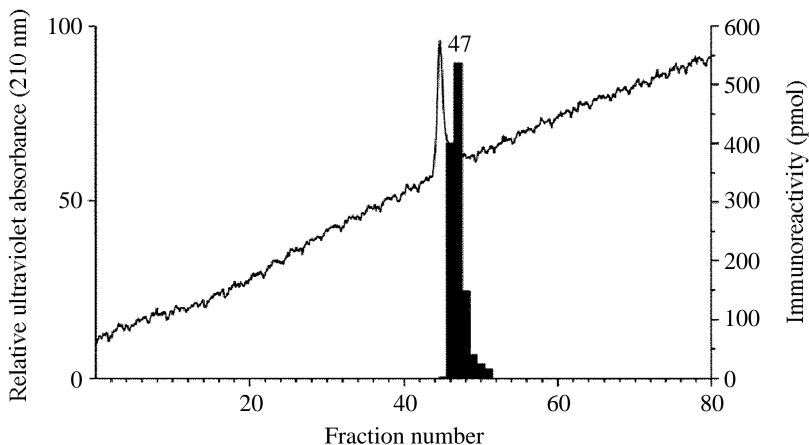

Fig. 4. Ultraviolet absorbance traces and immunoreactivity (EFLRIamide antiserum) profile of purified pQDPFLRIamide from the fourth and final purification step. The immunoreactive fractions from the previous HPLC run were loaded onto the column and eluted with gradient 4 as described in Materials and methods. There is a lag of approximately 30–60s (1–2 fractions) between the ultraviolet detector and the fraction collector. The most immunoreactive fraction (47) was sent to T. D. Lee for FABms-ms analysis.

mouse-PAP (both at 1:100), rinsed and visualised with DAB/H_2O_2 as for the biocytin staining.

Controls for specificity of staining

Some ganglia were treated according to the usual protocol, except that either the primary antiserum was omitted or a primary antiserum that had been preincubated with peptide for 24h was used. Samples of each antiserum were preincubated with FMRFamide, pQDPFLRIamide or SGYLAFPRMamide at 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} mol l^{-1}.

Results

pQDPFLRIamide can be purified from the central nervous system

An acetone extract of 50 circumoesophageal rings (brains) was processed and then purified in four HPLC fractionations, as described in the Materials and methods section. The purification was monitored by RIA with the EFLRIamide antiserum.

A single immunoreactive peak was detected in the RIA after the fourth and final HPLC run (Fig. 4). This immunoreactive peak, which lagged behind the single ultraviolet absorbance peak by 30–60s (the time required for the sample to travel from the detector to the fraction collector), could not be sequenced by Edman degradation; the presumed blocked amino terminal precluded this approach.

The structure of the peptide was determined by mass spectral analysis with a triple-sector quadrupole mass spectrometer (FABms-ms). The measured monoisotopic mass (m/z 870.5) of the protonated molecule was consistent with that calculated for the amino acid sequence pQDPFLRIamide, the sequence, including the presumed C-terminal

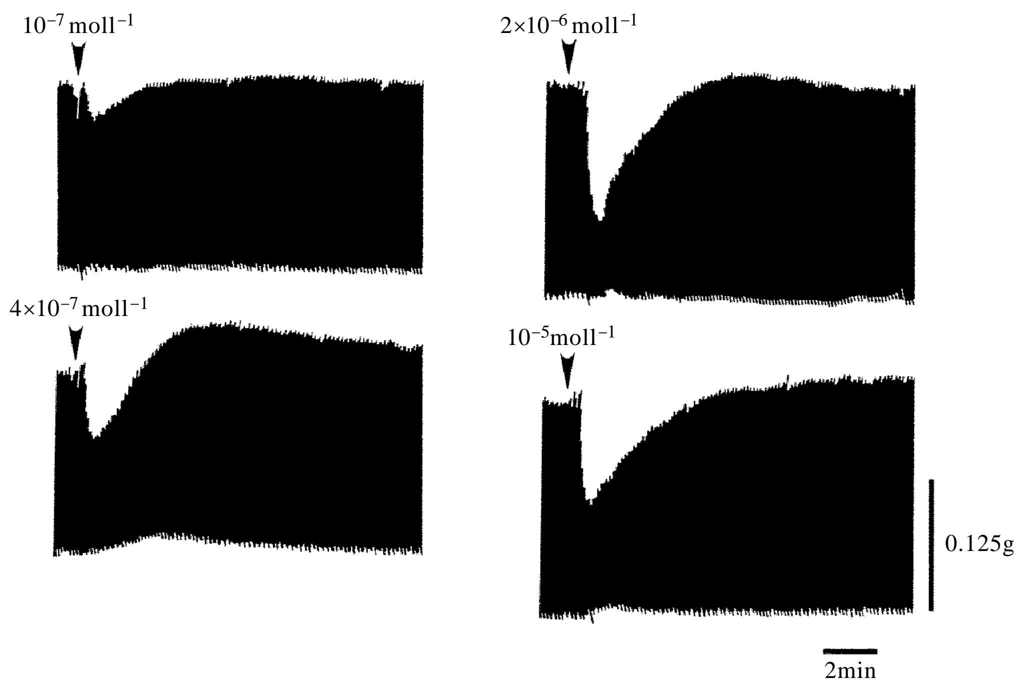

Fig. 5. Responses of the isolated ventricle to increasing doses of pQDPFLRIamide. 400 μl of saline containing the indicated dose was injected at the arrowheads.

amidation, that was inferred from the cDNA encoding the precursor. A spectrum of the fragment ions, derived from the protonated molecular ion by low-energy collisions in an argon gas cell, contained sufficient sequence information to confirm the structure of the peptide. Nearly all the members of the A, B and Y″ series (Roepstorff and Fohlman, 1984) were present as prominent ions in the fragment ion spectrum. Synthetic pQDPFLRIamide also co-eluted with fraction 46 from the last purification run, further confirming the sequence.

pQDPFLRIamide has a biphasic effect on the isolated ventricle

Ventricles were isolated from dormant snails and prepared for perfusion, and the effects of pQDPFLRIamide were tested according to the method of Payza (1987), as subsequently modified by Price *et al.* (1990). The predominant action of pQDPFLRIamide on the ventricle was to reduce the amplitude of the beat (Fig. 5); the threshold was between 4×10^{-8} and 10^{-7} mol l^{-1}. This inhibitory response was sometimes followed by an increase in beat amplitude (see response to 4×10^{-7} mol l^{-1} peptide in Fig. 5). Maximal inhibition occurred at about 2×10^{-6} mol l^{-1}; higher doses caused equal, or even diminished, responses. Diastolic tone was elevated in some preparations, but there was no obvious effect on the beat frequency.

The four antisera have different binding affinities in an RIA

We used RIA to quantify the peptides in fractionated extracts of cardiovascular and

nervous tissues and, as an aid to interpretation, investigated the binding affinities and specificities of the four antisera employed in these assays. The binding of each antiserum to a large set of analogues was examined and the IC_{50} values found in at least three determinations were averaged. The averages are displayed in Fig. 6; structurally similar peptides are grouped together in each panel.

The S253 antiserum (raised against YGGFMRFamide) (Fig. 6A) and the Q2 antiserum (raised against pQDPFLRFamide and DDPFLRFamide) (Fig. 6B) both had their highest affinities for the naturally occurring FMRFamide-like heptapeptides. The affinity for YGGFMRFamide, a synthetic heptapeptide analogue, was also greater than for FMRFamide itself. Oxidation of the methionine in FMRFamide (oxFMRFamide) increased the IC_{50} values: fourfold in the case of S253 and 100-fold for Q2. Removing the amide drastically reduced the affinity of the antisera for the peptide; indeed, the Q2 antiserum could not detect even 50nmol of FMRF and FLRF in 50 μl – the entire sample volume of the assay. Neither of the FMRFamide antisera bound particularly well to the two SCPs, but both antisera recognised all of the FMRFamide analogues endogenous to *H. aspersa*, though S253 had a greater affinity for SDP-, NDP- and pQDP-FLRFamide than for the tyrosine heptapeptides (NDPYLRFamide and SEPYLRFamide). Both antisera required a terminal -Famide for effective binding (compare pQDPFLRFamide and pQDPFLRIamide).

The S253 antiserum had a greater affinity for the putative peptides ENNNGYIRFamide and YGWAEGDTTDNEYLRFamide than it did for the tyrosine heptapeptides. The relatively high affinity of this antiserum for these two peptides may be due in part to the absence of a proline (proline introduces a bend in the peptide and is missing from YGGFMRFamide, the peptide against which S253 was raised).

The Q2 antiserum appeared to bind best to peptides ending -L*x*Famide (where *x* is any single residue); both isoleucine (compare ENNNGYIRFamide and YGWAEGDTTDNEYLRFamide) and methionine (compare FMRFamide with FLRFamide) were poor substitutes for leucine. This result was expected, since the Q2 antiserum was used to isolate four peptides with the terminal sequence -L*x*Famide (Elphick *et al.* 1991; Díaz-Miranda *et al.* 1992).

The affinity of the EFLRIamide antiserum (raised against EFLRIamide) for pQDPFLRIamide was 5000 times greater than it was for any of the other peptides examined (Fig. 6C). In particular, replacing the C-terminal isoleucine with a phenylalanine residue drastically inhibited binding (compare pQDPFLRIamide and pQDPFLRFamide).

The polyclonal SCP_B antiserum (raised against SCP_B, MNYLAFPRMamide) showed a similarly marked preference for the two SCPs over the FMRFamide analogues (Fig. 6D).

The EFLRIamide antiserum binds to peptides ending in -RFamide in a non-competitive dot-blot assay

The binding properties of the three antisera used in immunohistochemistry (i.e. EFLRIamide raised against EFLRIamide, Weber's raised against FMRFamide, and a

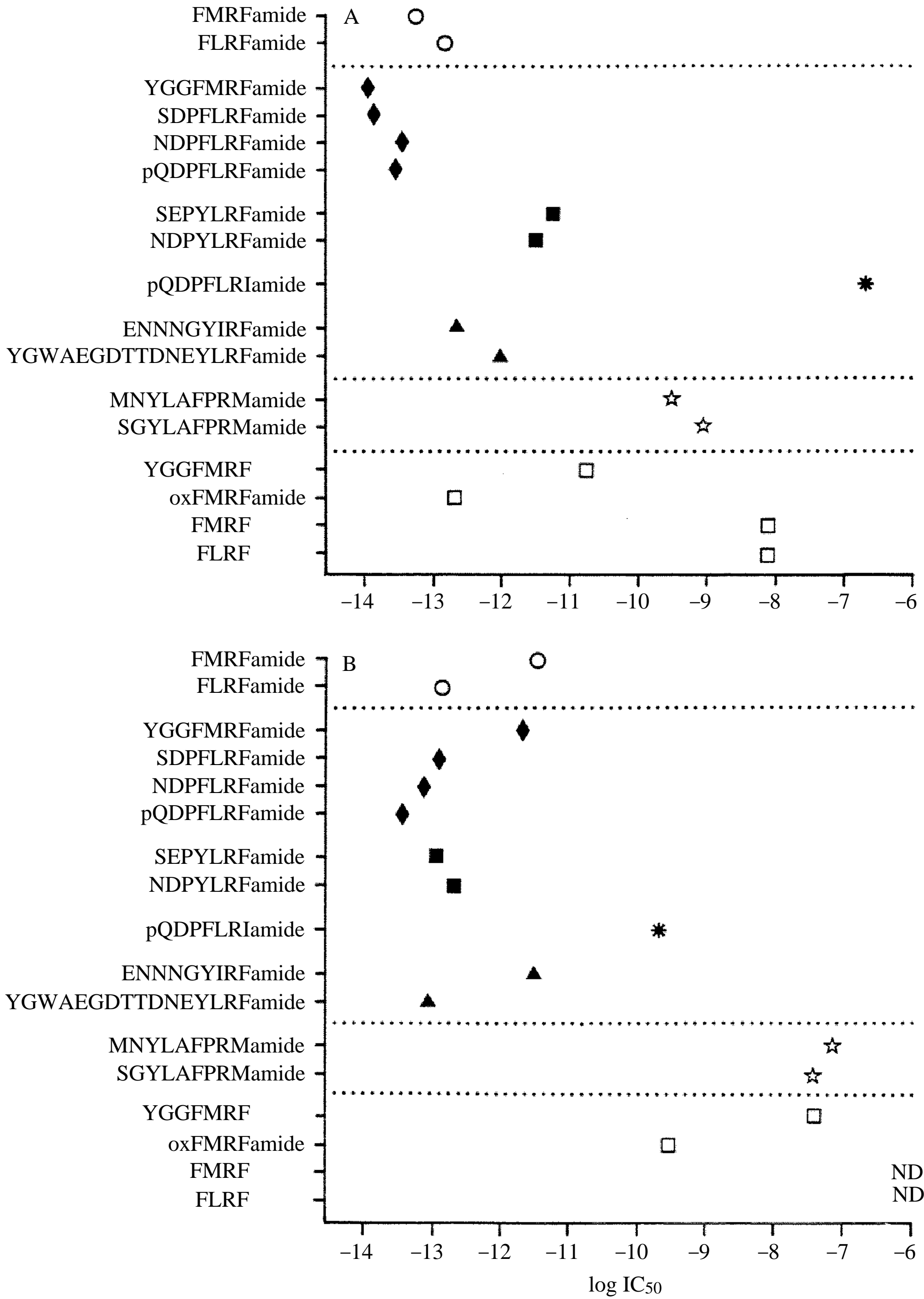

Fig. 6A and B. For legend see p. 218

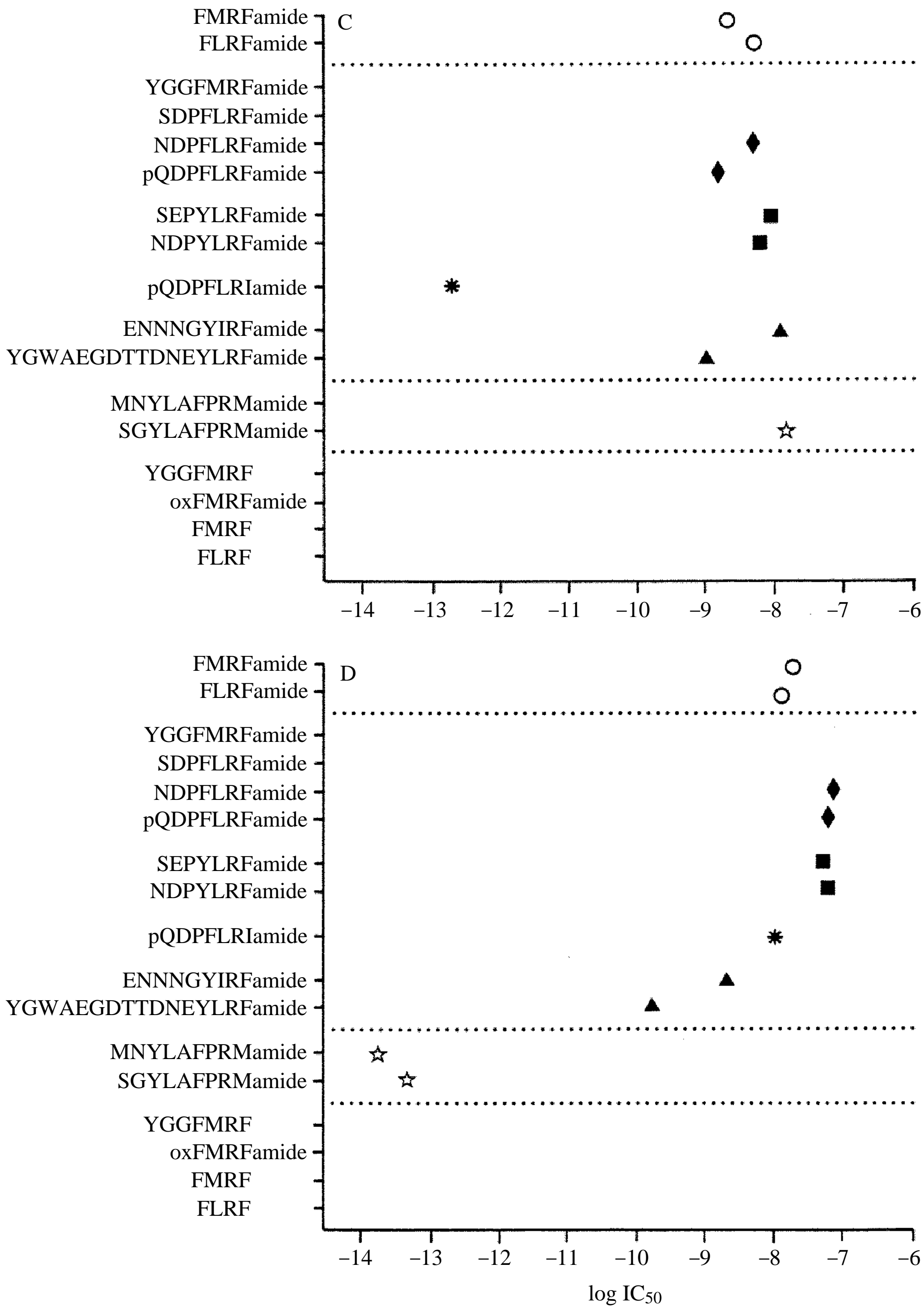

Fig. 6C and D. For legend see p. 218

Fig. 6. IC_{50} values of the various peptides for the four antisera used in the RIAs. The IC_{50} values (in $mol\,l^{-1}$) from a minimum of three experiments were averaged for each peptide. The standard errors were small, but are not shown. The peptides are listed in the same order for each antiserum; this is not necessarily the order of potency. Structurally similar peptides are grouped and represented by the same symbol. The dotted lines delineate products of a single gene (with the exception of YGGFMRFamide and the bottom four peptides). The lack of a symbol indicates that the peptide was not tested. ND, 50 μl of $1\,mmol\,l^{-1}$ peptide was not detected. (A) S253 antiserum with trace ^{125}I-labelled pQYPFLRFamide; (B) Q2 antiserum with trace ^{125}I-labelled pQYPFLRFamide; (C) EFLRIamide antiserum with trace ^{125}I-labelled YPFLRIamide; (D) SCP_B antiserum with trace ^{125}I-labelled SCP_B.

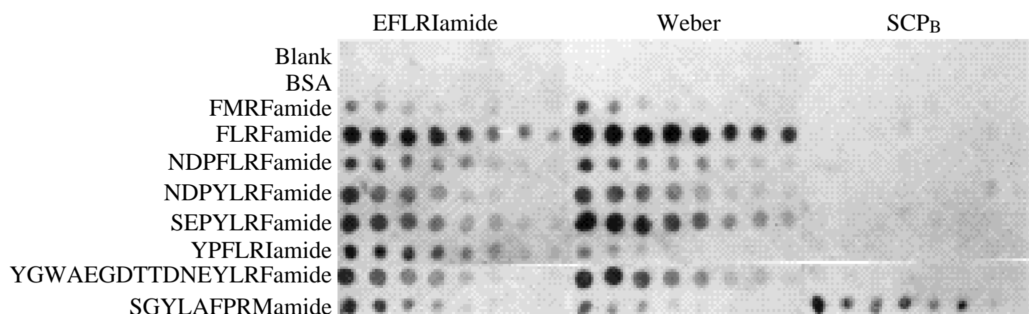

Fig. 7. An autoradiograph of a dot-blot assay of the binding of the three antisera used in immunohistochemical staining to the endogenous peptides. The three panels are identical in their peptide content. The peptides were conjugated to BSA through their N terminal ends, but this required an unprotected N terminal. Thus, pQDPFLRFamide and pQDPFLRIamide could not be used. Instead, NDPFLRFamide and pQDPFLRFamide were assumed to have similar binding affinities to each of the antisera, as were pQDPFLRIamide and YPFLRIamide.

monoclonal SCP_B raised against SCP_B) were investigated in a non-competitive environment. The most striking result was the non-specificity of the EFLRIamide antiserum binding in the dot-blot assay (Fig. 7), especially when contrasted with its high specificity in the RIA. Indeed, the EFLRIamide antiserum had the least specificity, binding to most of the peptides. Weber's antiserum had a lesser affinity for SGYLAFPRMamide and minimal affinity for YPFLRIamide, and the SCP_B monoclonal bound only to SGYLAFPRMamide.

Since the degree of conjugation could not be quantified, the different peptides could not be dotted onto the initial columns in known (i.e. equal) amounts. Thus, the binding affinities of any one antiserum to the various peptides must be compared cautiously. In contrast, each of the three 8×10 blots had identical peptide contents, so the relative affinities of the different antisera for any particular peptide are comparable.

The peptides are differentially distributed throughout the cardiovascular system

Acetone extracts of selected tissues within the cardiovascular and central nervous systems were fractionated by HPLC and analysed by RIA. Figs 8–12 show the distributions of the peptides within these systems. The brain contains all eight FMRFamide analogues and both SCPs (Fig. 8), as does the visceral nerve trunk (Fig. 9).

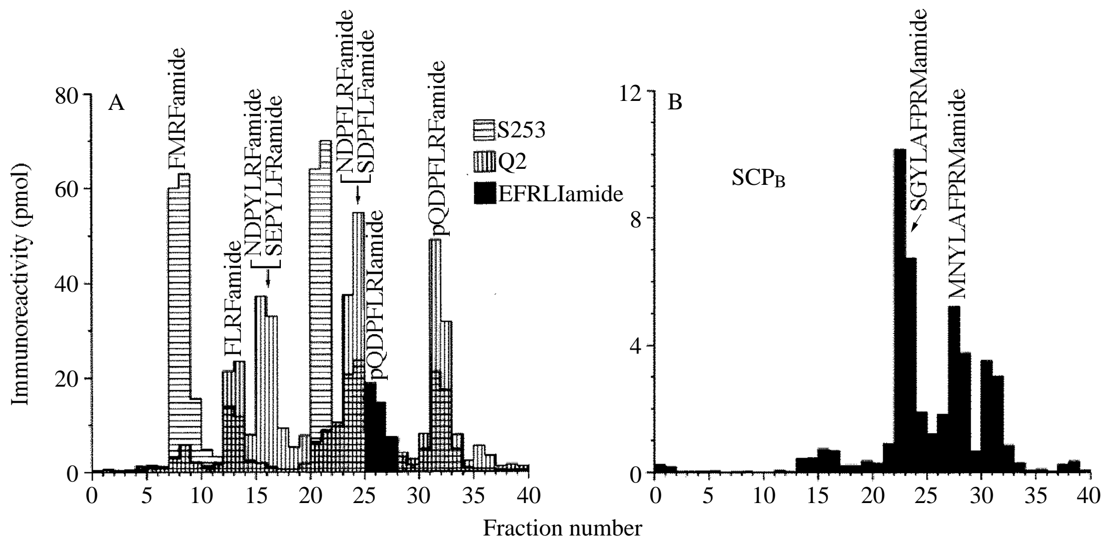

Fig. 8. RIA analysis of HPLC fractions of acetone extracts of the circumoesophageal ring. (A) Immunoreactivity with three different RIAs for FMRFamide-related peptides. The peaks identified with the S253 antiserum are superimposed upon those identified with the Q2 antiserum. The peak identified with the EFLRIamide antiserum at the position of pQDPFLRIamide is also shown. (B) The same fractions analysed with SCP$_B$ antiserum. All ten peptides are present. The elution positions of relevant synthetic peptides are indicated and the values have been adjusted to indicate the immunoreactivity of one ring.

The visceral nerve, in addition to innervating the gut, liver and gonads, also gives rise to the cardiac nerve, which innervates the heart (Fig. 2). Unfortunately, the cardiac nerve is never isolated from other nerves. As it branches off the visceral nerve, it is accompanied by the innervation to the spermatheca (⚥ Fig. 2C), aorta and gut. Thus, its complement of peptides could not be analysed and we cannot say whether it contains all ten identified endogenous cardioactive peptides or just one or two of the three sets (tetrapeptides, heptapeptides or SCPs) of these peptides.

The proximal (Fig. 10A,B) and distal (Fig. 10C,D) lengths of the aorta (see Fig. 2B) contain all ten peptides, though the heptapeptide content of the proximal segment is ten times that of the distal segment (compare the pQDPFLRIamide levels in the two segments, Fig. 10A,C). Both segments were taken from the same five animals and had similar masses, suggesting that this difference is significant. The SCPs and both tetrapeptides are present in the heart, but the heptapeptides are notably absent from this tissue (Fig. 11). As the third large peak recognised by the S253 antiserum in the heart is not recognised by the Q2 antiserum, it cannot be one of the identified FMRFamide analogues; the identified peptides that elute in this region bind to Q2 as well as, if not better than, they bind to the S253 antiserum.

Only low concentrations of two known peptides were detected in the haemolymph

The RIA analysis of the fractionated haemolymph extracts (Fig. 12) was quite different

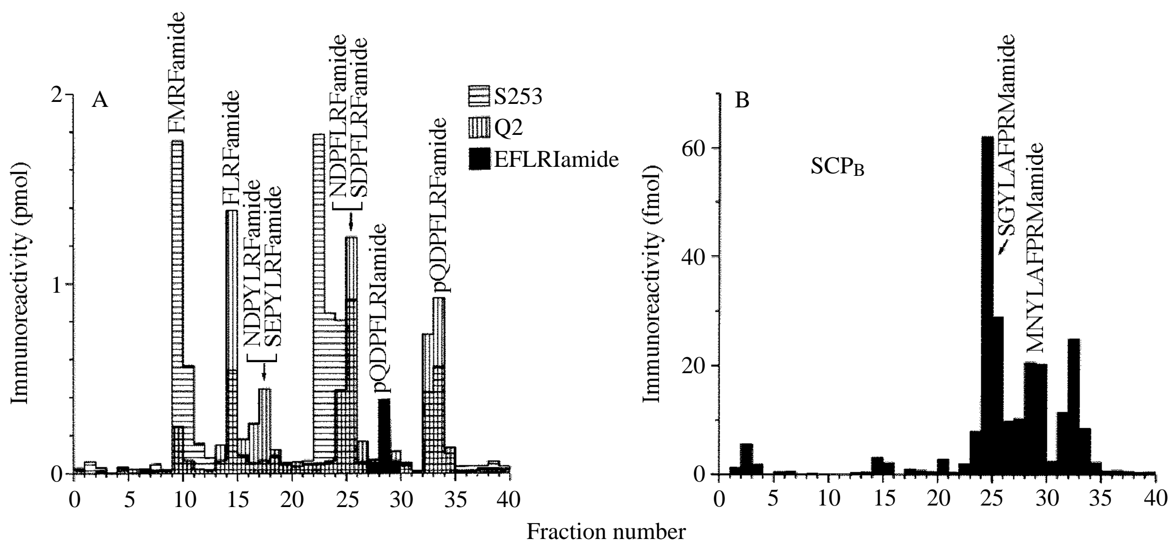

Fig. 9. RIA analysis of HPLC fractions of acetone extracts of the visceral nerve trunk. Labelling as in Fig. 8. All ten peptides are present. The values have been adjusted to indicate the immunoreactivity of one trunk.

from that of the other tissues (e.g. compare with Fig. 9). The single highly immunoreactive region (fractions 32–36), detected only by antiserum S253, includes the elution position of pQDPFLRFamide. Note, however, that the most immunoreactive fractions within this region (33 and 34) are particularly unreactive with Q2. Since pQDPFLRFamide binds equally well to antisera S253 and Q2 (Fig. 6A,B), the immunoreactive material eluting in fractions 33 and 34 cannot be pQDPFLRFamide.

The small immunoreactive peak, only slightly above background, at about fraction 32 might be pQDPFLRFamide, for the Q2 and S253 immunoreactivities in this fraction are similar. Moreover, the single small peak of immunoreactivity with the EFLRIamide antiserum in fractions 27 and 28 (the approximate elution position of pQDPFLRIamide) is about the same size as that in fraction 32; and if pQDPFLRIamide and pQDPFLRFamide were in the haemolymph, they should, on the basis of the gene sequence (Lutz *et al.* 1990), be present at similar levels.

The fractions contained no other peaks of Q2 and S253 immunoreactivity indicative of the remaining four heptapeptides. These peptides are unlike pQDPFLRFamide and pQDPFLRIamide, however, in that their N terminals are unprotected and thus they are more susceptible to degradation by enzymes in the haemolymph.

Could the small putative peaks of pQDPFLRFamide and pQDPFLRIamide represent cardioactive levels of those peptides in the haemolymph? To address this issue, 4pmol of pQDPFLRIamide was added to haemolymph and saline (i.e. $1\,\mathrm{nmol\,l^{-1}}$) before they were fractionated by HPLC and analysed by RIA. The resultant pattern of immunoreactivity (not shown) was similar to that of the unadulterated haemolymph, except that the immunoreactivity increased slightly above the background level for fractions 28 and 29 (the elution position for pQDPFLRIamide). This result suggests that threshold levels

(1 nmol l^{-1}) of peptide are present in the haemolymph and are just detectable by our procedures.

The tetrapeptides are released from the heart in a calcium-dependent manner

Calcium-dependent release of FMRFamide-related peptides from the heart was readily demonstrated; details of the experiment are shown in Fig. 13. A tenfold increase in the

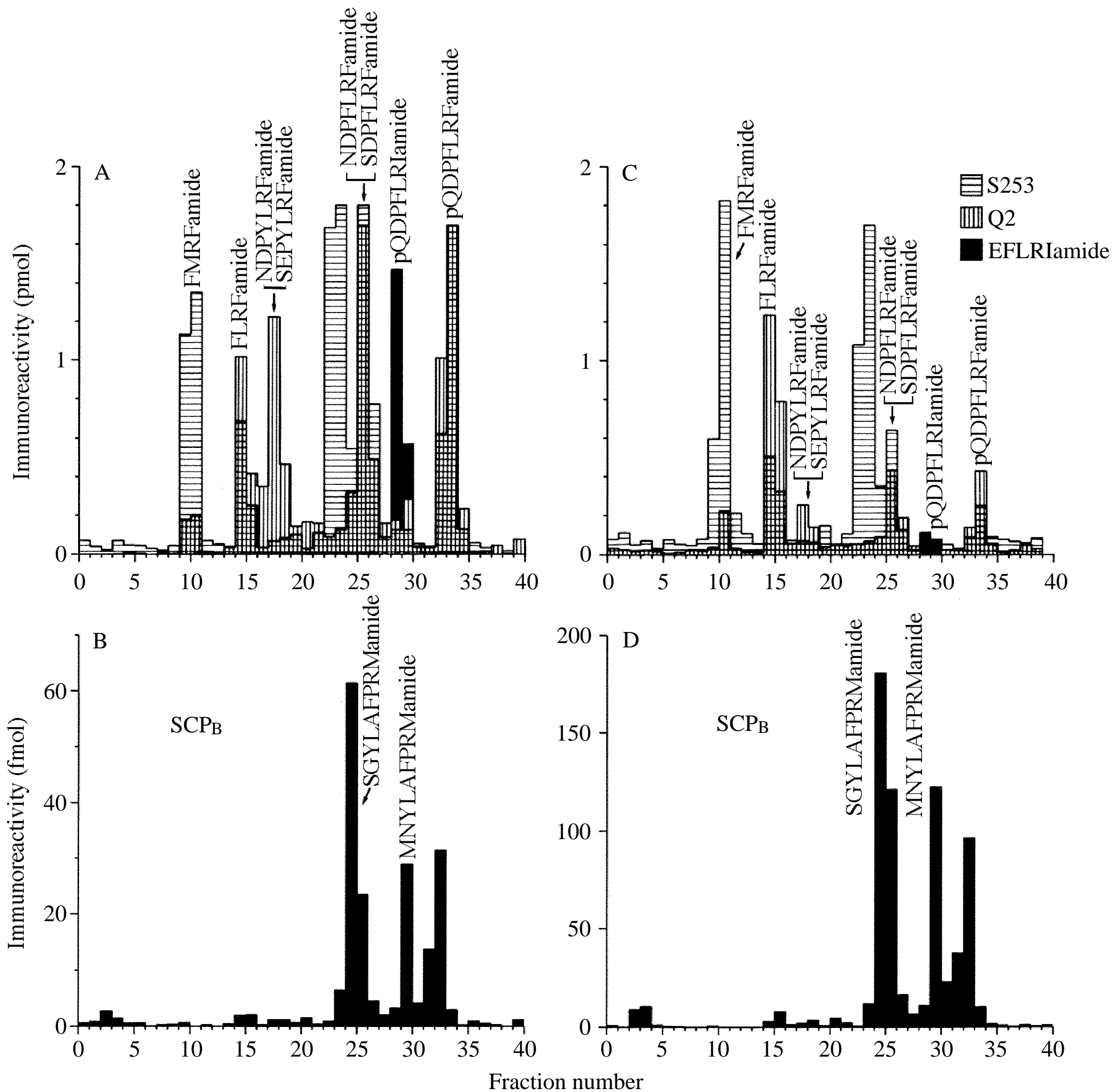

Fig. 10. RIA analysis of HPLC fractions of acetone extracts of the proximal (A,B) and distal (C,D) segments of the anterior aorta. (A,C) Immunoreactivity with three different RIAs for FMRFamide-related peptides. (B,D) The same fractions analysed with SCP_B antiserum. Labelling as in Fig. 8. All ten peptides appear to be present. The values have been adjusted to indicate the immunoreactivity of one aorta.

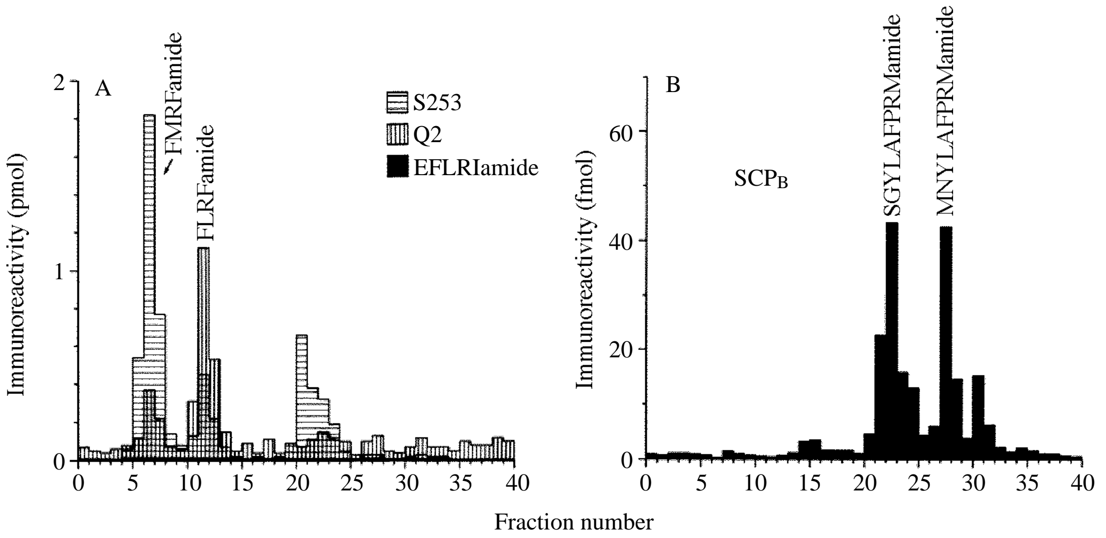

Fig. 11. RIA analysis of HPLC fractions of acetone extracts of the heart. (A) Immunoreactivity with three different RIAs for FMRFamide-related peptides. No peaks of immunoreactivity were identified with the EFLRIamide antiserum; this is indicated by a solid black line at the base of A. Note that no immunoreactive peaks have been detected by the S253 and Q2 antisera at the elution positions for the heptapeptides. (B) The same fractions analysed with SCP_B antiserum. The elution positions of the tetrapeptides and SCPs are indicated and the values have been adjusted to indicate the immunoreactivity of one heart.

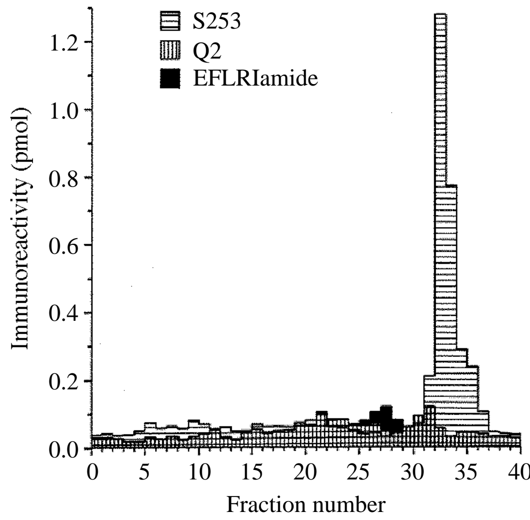

Fig. 12. RIA analysis of HPLC fractions of acetone extracts of the haemolymph. Immunoreactivity with three different RIAs for FMRFamide-related peptides. The three profiles obtained with S253, Q2 and EFLRIamide antisera are superimposed upon each other. The values have been adjusted to indicate the immunoreactivity of 1ml of haemolymph.

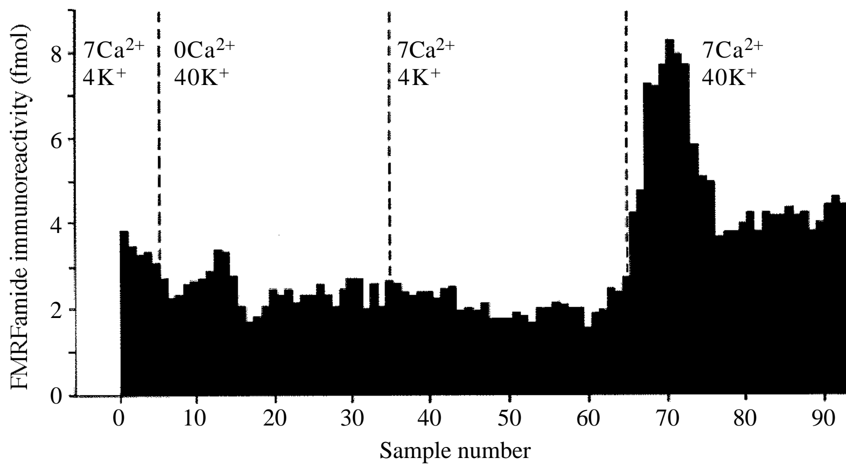

Fig. 13. Demonstration of the release of FMRFamide peptides from the heart. The calcium and potassium contents (in mmol l^{-1}) of the saline perfusing the tissue at the time of collection of each drop are indicated along the top of the figure. The composition of the first saline is the 'normal' composition. Each bar of the histogram represents the FMRFamide immunoreactivity of a 40 μl drop.

potassium concentration of the perfusion saline had no effect when the calcium content was nominally zero, but when the calcium was restored to 'normal' levels, the perfusion of a high potassium concentration effected the release of FMRFamide-related peptides.

The cardiac innervation

The visceral nerve sends atrial and ventricular branches to the heart (see Fig. 2); biocytin, an intracellular marker, was used to trace these two innervations. The atrial branch enters the atrium, crosses the atrial-ventricular (a-v) junction and extends into the ventricle (Fig. 14A,B). Within the atrium, the innervation is a dense, homogeneous network associated with the muscular wall of this chamber. Only a few neuronal components of this network cross the a-v junction to the ventricle, where they branch diffusely; varicosities can also be seen (indicated by arrows). Even after a 7-day incubation in biocytin, this innervation is restricted to the half of the ventricle closest to the atrium.

The ventricular branch of the visceral nerve innervates the aorta and ventricle (Fig. 14C). This innervation of the heart is more restricted than that from the atrial branch. It provides a diffuse innervation to the neck of the ventricle and peters out as it ascends into the body of the ventricle, though one or two branches do extend across the a-v junction into the atrium (indicated by an arrowhead in the atrium).

Immunohistochemical staining of the cardiovascular system reveals a diffuse network of nerve fibres

Having quantified the peptides present within the different sections of the cardiovascular system, we attempted to visualise their patterns of distribution by immunohistochemistry. Immunoreactive fibres (examples indicated by arrowheads) were

found in all regions, though the pattern varies with the antiserum employed (Fig. 15). The portion of the ventricle closest to the atrium has the lowest density of neuronal staining with all three antisera. This region of mainly diffuse background staining (indicated by an asterisk in Fig. 15A) presumably represents innervation primarily by the atrial branch of the cardiac nerve.

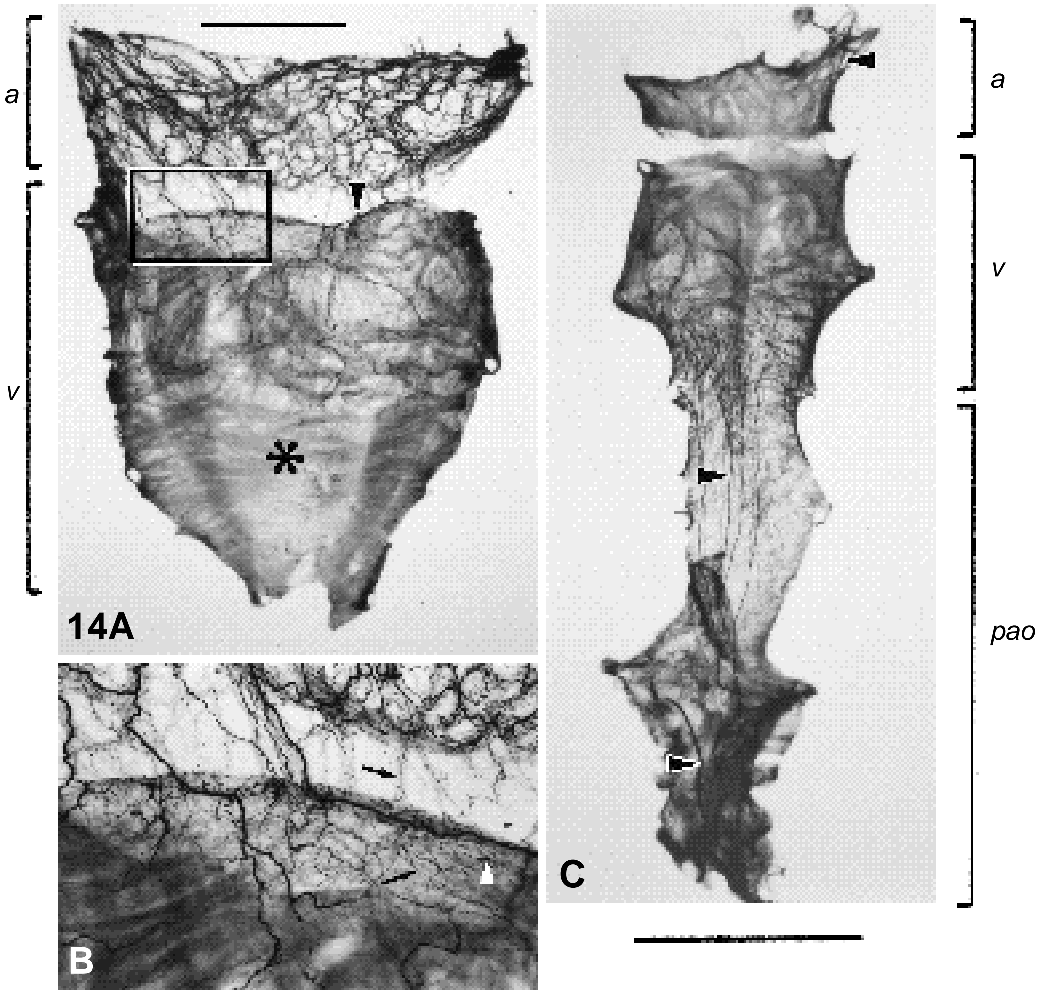

Fig. 14. A biocytin fill of the two branches of the visceral nerve innervating the heart. The photographs are whole mounts. (A) A fill of the atrial branch. Only the heart itself is shown. A region of background staining is indicated by an asterisk for comparison. (B) A higher magnification of the atrium and upper ventricle boxed in A. Neuronal cell bodies are indicated by the arrowheads in A and B and varicosities are indicated by arrows in B. (C) A fill of the ventricular branch. The heart and proximal aorta are shown. Filled neuronal fibres are indicated by the arrowhead in the aorta. A filled axon that has crossed the atrial-ventricular junction into the atrium is indicated by the arrowhead in the atrium. *a*, atrium; *v*, ventricle; *pao*, proximal aorta. Scale bars, 1mm.

The SCP_B antiserum produces the most dense staining, revealing a fine network weaving throughout the heart and aorta (Fig. 15C). With all three antisera, some staining appears to follow the cardiac trabeculae, indicating that these fibres are on, or just below, the lumenal surface of the heart, while other fibres are deeply embedded within the

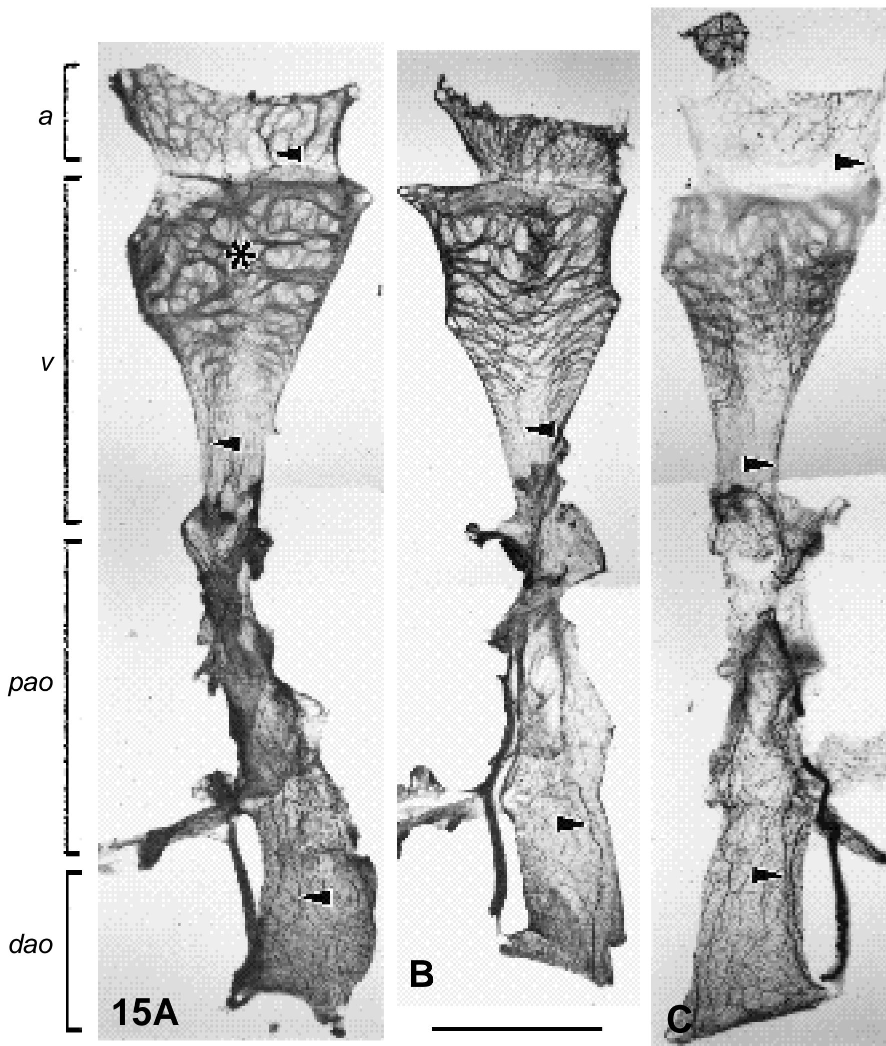

Fig. 15. Immunohistochemistry of the heart and aorta between the heart and circumoesophageal ring. The photographs are whole mounts treated with (A) Weber's antiserum (raised against FMRFamide), (B) EFLRIamide antiserum, and (C) SCP_B antiserum. Some densely stained pulmonary vein remains attached to the atrium in C. Some immunoreactive fibres are indicated by arrowheads; a region of background staining (upper ventricle) is indicated by an asterisk in A. *a*, atrium; *v*, ventricle; *pao*, proximal aorta; *dao*, distal aorta. Scale bar, 2mm.

cardiac tissue. The fibres appear at all levels in the aorta wall, innervating not only the outer longitudinal muscle layer but also the inner circular muscle.

The heptapeptide levels in acetone extracts of the proximal segment of the aorta (Fig. 10A) are tenfold those of the distal segment (Fig. 10C) (compare the pQDPFLRIamide levels), but the density and pattern of the immunohistochemical staining in these two regions were similar (Figs 15A,B). The peptide concentration within the neurones of the distal segment, though low, may saturate the antibody binding so that no further increase in the staining density of the proximal segment is possible.

Controls for specificity of staining

Staining with the SCP_B monoclonal antiserum was blocked by absorption with 10^{-7} mol l^{-1} SGYLAFPRMamide, but it persisted in the presence of 10^{-8} mol l^{-1} SGYLAFPRMamide, 10^{-4} mol l^{-1} FMRFamide and 10^{-4} mol l^{-1} pQDPFLRIamide. Similar results were obtained with the EFLRIamide antiserum; staining was blocked following preincubation with 10^{-7} mol l^{-1} pQDPFLRIamide, but persisted in the presence of 10^{-8} mol l^{-1} pQDPFLRIamide, 10^{-4} mol l^{-1} SGYLAFPRMamide and 10^{-4} mol l^{-1} FMRFamide. Weber's antiserum stained the tissue despite preincubation with 10^{-4} mol l^{-1} SGYLAFPRMamide, 10^{-4} mol l^{-1} pQDPFLRIamide and even 10^{-5} mol l^{-1} FMRFamide. No specific staining was observed when the primary antisera were omitted. These results are not shown.

Discussion

The putative peptide pQDPFLRIamide is, indeed, processed

We have demonstrated that pQDPFLRIamide, predicted from the gene encoding its precursor (Lutz *et al.* 1990), is found in the circumoesophageal ring and other tissues of *Helix aspersa* and is, therefore, processed. Since virtually all RIAs and bioassays for FMRFamide-like peptides share a common specificity for a C-terminal phenylalanine amide, none of them would have detected a peptide with a C-terminal isoleucine amide. The gene encoding the tetrapeptides FMRFamide and FLRFamide in *Lymnaea stagnalis* also contains sequences for the putative peptides EFLRIamide and pQFYRIamide (Linacre *et al.* 1990), but the processing of these peptides has not been confirmed. Nevertheless, an antiserum raised against EFLRIamide (Santama, 1992) aided in the identification of pQDPFLRIamide in *H. aspersa*. Hence, the genetic approach and subsequent findings are critical to the identification of such cryptic members of peptide families as pQDPFLRIamide.

The FMRFamide-related tetrapeptides may be acting as neurotransmitters in the heart

Among the peptides that we have tested on the heart (Price *et al.* 1990), the tetrapeptides are not very potent. Nevertheless, they are certainly present in the circumoesophageal ganglia, the visceral nerve trunk, the aorta and, especially, the heart. Further, the immunohistochemical evidence suggests that both branches of the cardiac nerve, which provide a diffuse innervation throughout the heart, contain the tetrapeptides. Finally, the calcium-dependent release of FMRFamide-immunoreactive material from

the heart suggests strongly that the tetrapeptides are released under physiological conditions. We therefore suggest that the tetrapeptides are synthesised in neuronal cell bodies in the circumoesophageal ganglia, are transported to the heart in axons running in the visceral nerve to the cardiac nerve, and are released from varicosities or terminals in close apposition to protected, postjunctional receptors on the cardiac muscle. The high concentrations of peptide around the receptors would explain the high threshold.

Three interpretations of the immunoreactivity to the EFLRIamide antiserum observed in the heart

The EFLRIamide antiserum, as used in the RIA, was specific for the C-terminal -Ile-amide and revealed no pQDPFLRIamide in the heart. In immunohistochemistry, however, it was quite unselective in its binding (in a dot-blot assay) and stained nerves in the heart as well as the other antisera. These observations can be interpreted as follows.

First, two small peaks of EFLRIamide immunoreactivity elute near the positions of the tetrapeptides (W. Lesser, unpublished data). Since the tetrapeptide transcript has not been fully sequenced in *H. aspersa*, it may, like that of *Lymnaea stagnalis*, include, at the 5′ end, one or more peptide sequences ending in -RIamide. Such products could be responsible for the EFLRIamide antigenicity observed in the heart.

Second, *H. aspersa* may possess additional peptides with an -RIamide at the C terminal, which are encoded by neither FMRFamide transcript, but which, nevertheless, innervate the heart. For example, TSSFVRIamide has been purified from *Helix pomatia*, SPSSFVRIamide and APSNFIRIamide from *Achatina fulica* (Ikeda *et al.* 1991) and LSSFVRIamide from *Fusinus ferrugineus* (Kuroki *et al.* 1992). The genes for these peptides have not been sequenced.

Finally, the dot-blot assay indicates that the EFLRIamide antiserum will bind to peptides ending in -RFamide as well as those ending in -RIamide. Thus, this antiserum, when applied directly to tissues, may be binding merely to the various peptides ending in -RFamide that are present in the heart.

We also conclude, more generally, that the structure–activity relationships of an antibody in a competitive assay (e.g. an RIA) are not necessarily transferable when the same antibody is used in a non-competitive assay (e.g. immunohistochemistry). The calibration of an antibody must be designed to match the experimental conditions and must be carried out anew when the conditions change.

The heptapeptides could be secreted into the haemolymph to act hormonally on the heart

Though its effect is inhibitory rather than excitatory, pQDPFLRIamide is, like the other heptapeptide analogues of FMRFamide, a potent cardioactive agent. However, the potency of the heptapeptides on the heart is at odds with their unexpected and complete absence from the heart – a unique distribution among identified SCP- and FMRFamide-related peptides. Therefore, if their cardioactivity is physiological, the heptapeptides must be transported to the heart in the haemolymph as neurohormones.

But where is the release site? As the aorta passes through the fused suboesophageal ganglia, its branches ramify into the connective tissue sheath, eventually forming an

almost continuous haemolymph-filled space lying just above the surface of the nervous tissue (Pentreath and Cottrell, 1970). It is possible that the heptapeptides are secreted from the brain into the haemolymph, to travel in the circulation to the heart. There they would bind to receptors that would be relatively well exposed to the haemolymph and particularly sensitive to the heptapeptides.

If FMRFamide-like heptapeptides released from the brain are affecting the heart, then haemolymph sampled between the brain and the heart should have suprathreshold levels of these peptides. In fact, the mean concentration of pQDPFLRFamide in the haemolymph of *Helix aspersa* was previously reported to be 1–10nmol l^{-1} (Lehman and Price, 1987); these samples were taken from the aorta, i.e. as the haemolymph was leaving the heart. In our investigation, haemolymph taken from the vena magna contained peaks of heptapeptide immunoreactivity that were much smaller than those found earlier, though the data were consistent with nanomolar, or slightly lower, haemolymph levels. Thus, a site much closer to the heart, such as the pulmonary vein leading to the atrium, could be another neurohaemal organ in the snail, and this possibility is under consideration.

The tetrapeptide and heptapeptide analogues of FMRFamide may act as transmitters in the aorta

The heptapeptides (as well as the tetrapeptide FMRFamide analogues) have been identified in the aorta. Immunohistochemical staining was associated with both muscle layers in the aorta, the inner circular and the outer longitudinal, though this finding is in contrast to that of Griffond *et al.* (1986), who used a commercial FMRFamide antiserum and observed staining only in the outer longitudinal layer. In any event, the FMRFamide-like peptides, in addition to strengthening the heart beat, could be influencing the regulation of haemolymph flow, at least in the aorta if not elsewhere in the general circulation.

The SCPs appear to be cardioregulatory transmitters

Both of the known SCPs, SGYLAFPRMamide and MNYLAFPRMamide, have been extracted from the brain, the visceral nerve trunk and the heart. Immunohistochemical staining with SCP_B antiserum is diffuse rather than localised to any particular region of the heart. Moreover, the SCP levels measured by HPLC fractionation of heart extracts and subsequent RIA are very low (<100fmol per heart), precluding the demonstration of calcium-dependent release, and the isolated heart is more sensitive to exogenously applied SCPs than to similarly applied FMRFamide analogues. The SCPs could, therefore, be local cardiac hormones, released non-synaptically from a diffuse innervation and binding to exposed hormonal receptors. Alternatively, they could be binding with a high affinity and efficacy to protected postsynaptic receptors.

The cardiovascular system is under the integrated control of many influences

For many decades we have known that molluscan hearts can be influenced by the classical transmitters 5-hydroxytryptamine and acetylcholine. During the 1960s,

evidence accumulated suggesting that additional substances contribute to the modulation of cardiac activity. A number of these substances have since been identified, including the endogenous peptides described here and by Price *et al.* (1990). We now know that these peptides function not only as transmitters but also as hormones and that they do not act alone but in combination. Thus, the *H. aspersa* heart is stimulated by lower concentrations of the SCPs and -RFamide peptides and is inhibited by higher doses of these same peptides and, also, by pQDPFLRIamide. However, the state of cardiac activity at any one time is governed by the combined effects of all these neurohumours and, in addition, the cardiac output will be modulated indirectly by the direct action of these agents on the aorta.

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