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SEVEN FMRFamide-RELATED AND TWO SCP-RELATED CARDIOACTIVE PEPTIDES FROM *HELIX*

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Summary

Pulmonate snails have a more complex array of cardioexcitatory peptides than other molluscs, and *Helix* has a more complex array than most other pulmonates. Since a full characterisation of the cardioexcitatory peptides is necessary for an understanding of physiology, we sought to identify the members of two families of such peptides – the small cardioactive peptides (SCPs) and the FMRFamide-related peptides (FaRPs) – from *Helix aspersa*. We characterised the peaks of immunoreactivity from HPLC both by their elution times and by their molecular weights as determined by fast atom bombardment mass spectrometry (FABms). These two criteria, used in parallel, facilitated our identification of several known peptides: MNYLAFPRMamide, identical to SCP_B of *Aplysia*; two tetrapeptide FaRPs, FMRFamide and FLRFamide; and three heptapeptide FaRPs, NDPFLRFamide, SDPFLRFamide and pQDPFLRFamide. Of these peptides, only FMRFamide and pQDPFLRFamide have previously been reported from *Helix*. We also discovered an additional SCP and two novel FaRPs and sequenced them. The SCP is Ser-Gly-Tyr-Leu-Ala-Phe-Pro-Arg-Met-amide (SGYLAF-PRMamide), and the heptapeptide FaRPs are Asn-Asp-Pro-Tyr-Leu-Arg-Phe-amide (NDPYLRFamide) and Ser-Glu-Pro-Tyr-Leu-Arg-Phe-amide (SEPYLRF-amide). When these nine peptides were tested on isolated *Helix* ventricles, the SCPs were the most potent cardioexcitors, the heptapeptide FaRPs were next, and the tetrapeptides had the least activity.

Introduction

By 1960, the neurotransmitters in molluscan ganglia had been sufficiently well-investigated that some of the cardioexcitatory activity present in ganglia could no longer be ascribed to the major, known cardioexcitatory transmitter 5-hydroxytryptamine (5-HT). Among the studies suggesting the occurrence of unknown factors in ganglia, two of the earliest (Meng, 1960; Kerkut and Laverack,

Key words: FMRFamide, SCP, molluscan neuropeptides, peptide sequences, fast atom bombardment mass spectrometry, peptide oxidation.

1960) were conducted on species of *Helix*. Jaeger (1966) found that the novel activity in a related land pulmonate was inactivated by pronase and concluded that the active principle must be a peptide. Frontali *et al.* (1967) also attributed to peptides several peaks of cardioexcitatory activity separated chromatographically from clam and whelk ganglia.

Several of these putative molluscan cardioactive peptides have been identified and sequenced in the past 13 years. They fall into two families, the peptides related to FMRFamide and those related to the SCPs, and their distributions among the Mollusca and other phyla are under study (Price and Greenberg, 1989; Price *et al.* 1989). In this paper we look at the complement of cardioactive peptides in a single species, *Helix aspersa*, the relative potencies of these peptides as cardioactive agents and, ultimately, their roles (individually and in the aggregate) as regulators of the circulation in this snail.

The FMRFamide-related peptides

Price and Greenberg (1977) identified the tetrapeptide amide FMRFamide from clam ganglia. Shortly afterwards, in collaboration with Cottrell, they began to search for FMRFamide in *Helix aspersa* using the radula protractor muscle from a whelk as a bioassay. No authentic FMRFamide was found, but only related peptides that seemed to have a more potent effect on the *Helix* heart than FMRFamide itself (Cottrell *et al.* 1981). Abandoning the bioassay, they began to use radioimmunoassay (RIA) to follow the purification of these FMRFamide-like peptides. Eventually, one of them, pQDPFLRFamide, was sequenced, and FMRFamide itself was identified in *Helix*, but several peaks remained uncharacterised (Price *et al.* 1985).

Three natural heptapeptide analogues of pQDPFLRFamide were soon discovered in other pulmonate snails; their general sequence was: X-DPFLRFamide, where X could be serine, glycine or asparagine (Price *et al.* 1987*a*; Ebberink *et al.* 1987). Preliminary analyses of two immunoreactive peaks in *Helix* led to the speculation that one of these heptapeptides (NDPFLRFamide), as well as a novel analogue (NDPYLRFamide), was present (Price *et al.* 1987*b*). We will show that this speculation was only partially correct; each peak actually contains two peptides: SDPFLRFamide together with NDPFLRFamide and SEPYLRFamide together with NDPYLRFamide.

The SCPs

Even as the FMRFamide-like peptides of *Helix* were being investigated on the basis of their immunoreactivity, Lloyd (1978, 1980) was using the *Helix* heart to assay the cardioexcitatory activity of ganglion extracts from this snail. Though he found a peak of activity on Sephadex G-15 at an elution time similar to that of FMRFamide, FMRFamide is such a poor excitor of the *Helix* heart that it could not account for the activity. Therefore, Lloyd (1978) designated the activity peak 'SCP', an abbreviation for small cardioactive peptide. Later, he and his associates found a similar peak of activity in *Aplysia*, and showed that it is composed of two

related peptides, a nonapeptide sequenced from *A. brasiliana*, SCP_B (Morris *et al.* 1982), and an undecapeptide sequenced from *A. californica*, SCP_A (Mahon *et al.* 1985; Lloyd *et al.* 1987).

Since both *Aplysia* SCPs are potent excitors of the *Helix* heart (Lloyd *et al.* 1987), homologous peptides should be present in *Helix* itself. Indeed, immunoreactive SCP-like activity was previously found in *Helix*, and one peptide had an amino acid composition that suggested it was homologous but not identical to *Aplysia* SCP_A (Price, 1987).

We have developed RIAs that are highly specific for each peptide family (Price, 1987) and have used them to follow the purification of those members present in *Helix*. Here we report the complete identification and synthesis of the FMRF-amide- and SCP-related peptides so far found in the snail, as well as a description and evaluation of their activity on isolated ventricles. These data provide a foundation for further studies on the physiological roles of peptides in cardiovascular regulation.

Materials and methods

Preparation and purification of extracts

Whole snails (typically 25–75 animals, depending on size, to give about 40–50 ml) were crushed, their volume measured, and four volumes of acetone added. Ganglia were dissected from snails and dropped into a fourfold excess of acetone. The tissue was shaken in the acetone and put in the freezer for 12–48 h. The liquid was decanted, clarified by centrifugation and filtration through nylon filters (0.22 or 0.45 μm pore), and reduced in volume under a water aspirator vacuum with heating to 65 °C until all of the acetone was removed. An equal volume of aqueous 0.1 % trifluoroacetic acid (TFA) or water was added, and the liquid again centrifuged and/or filtered to remove any precipitate.

The clarified aqueous solution was pumped through a reverse-phase HPLC column (Waters NovaPak C18, 3.9 mm×150 mm, or Brownlee RP-18, 4.6 mm× 250 mm, at 2 ml min^{-1}; Brownlee RP-300, 2.1 mm×220 mm, at 0.5 ml min^{-1}). After all the extract had been pumped through, the column was washed with 0.1 % aqueous TFA until the absorbance fell about 90 % of the way back to the pre-injection level. The flow was then switched to the starting acetonitrile (ACN) composition. Once this solvent reached the detector (as shown by a rapid increase in the absorbance) an increasing, linear gradient of acetonitrile (slope of 0.8 % ACN min^{-1}) was started; 30 s fractions were collected, and a 2 μl sample was taken from each fraction for radioimmunoassay.

The one or two most immunoreactive fractions from each peak were pooled and injected back onto the same column with either *n*-butanol or isopropanol as the organic solvent, and again with 0.1 % TFA throughout. Other conditions were the same as those for the first HPLC run, except that the flow rate was reduced to 1.5 ml min^{-1} with the RP-18 column because of excessive pressure at 2 ml min^{-1} with these alcohols. The peak one or two most immunoreactive fractions from

each run were re-run through the ACN/TFA system. The two HPLC systems were used alternately until the peak was judged to be pure by its shape and by the correspondence between the amount of absorbance and immunoreactivity.

SCPs and oxidation

The SCPs – particularly SCP_B, which has two methionyl residues – oxidise readily, producing many different oxidation products (see Price, 1987, for examples). Furthermore, the SCP antiserum we use recognises the oxidised SCPs very well. Therefore, HPLC fractionation of SCP activity can give patterns that are difficult to interpret. One solution is to oxidise all the SCPs in the crude extract, but this can cause other problems. For example, Price (1987) previously reported an amino acid composition for a *Helix* SCP (reproduced here in Table 1) that had a lower than expected level of tyrosine, probably due to halogenation of the tyrosine by chloride present during oxidation. We have now avoided such complications by oxidising the peptide only after it is substantially pure, i.e. between the second and third HPLC steps. Since acidic conditions during oxidation favour the conversion of methionine to methionine sulphoxide over other products (see Neumann, 1967), we oxidised by direct addition of 50 $\mu l\ ml^{-1}$ of hydrogen peroxide (30 % solution) to the fraction containing 0.1 % TFA. The

Table 1. *Amino acid compositions of* Helix *SCP-like peptides*

Amino acid	SGYLAFPRMamide‡ (nmol)	Ratio	*Aplysia*§ SCP_A	MNYLAFPRMamide (nmol)	Ratio	*Aplysia*§ SCP_B
Glycine	1.163	1.16	1	0.14	0.28	0
Leucine	1.155	1.12	1	0.58	1.16	1
Proline	1.061	1.06	2	0.52	1.04	1
Arginine	1.040	1.04	2	0.57	1.14	1
Methionine†	1.007	1.00	1	1.00	2.00	2
Alanine	1.002	1.00	2	0.50	1.00	1
Phenylalanine	0.961	0.96	1	0.50	1.00	1
Serine	0.946	0.95	0	0.11	0.22	0
Histidine*	0.521	0.52	0	0.06	0.12	0
Tyrosine	0.281	0.28	1	0.50	1.00	1
Aspartic acid	0.183	0.18	0	0.50	1.00	1
Isoleucine	0.140	0.14	0	ND	0	0
Glutamic acid	0.121	0.12	0	0.11	0.22	0
Lysine	0.078	0.08	0	ND	0	0
Valine	ND	0	0	ND	0	0
Threonine	ND	0	0	0.10	0.20	0

ND, not detected (less than 0.05 nmol).

* Though this peak eluted at the retention time of histidine, we suspect that it is chlorotyrosine. The crude extract used to isolate SGYLAFPRMamide was oxidised, so the peptide's tyrosol residue may have been chlorinated.

† Value for methionine is sum of Met and Met sulphoxide.

‡ Data reprinted from Price (1987).

§ Values based on gene sequence (Mahon *et al.* 1985).

oxidation was terminated after 15 min by injection of the reaction mixture onto the HPLC.

Radioimmunoassay (RIA)

The basic method of the RIA for FMRFamide has been described (Price *et al.* 1987*a*), and we have used the same procedure and antiserum (S253) for much of the work reported here. More recently we raised another antiserum, Q2, by an initial immunisation with a conjugate of pQDPFLRFamide followed by boosting with a conjugate of DDPFLRFamide (see Price, 1982, for conjugation and immunisation methods). Iodinated pQYPFLRFamide served as the trace for both FMRFamide assays.

For the SCP assay we used a rabbit antiserum to SCP_B from H. R. Morris with iodinated SCP_B as the trace. The iodination method, buffer and assay were carried out exactly as for the FMRFamide assays.

Mass spectrometry

The method for obtaining FAB spectra of the FaRPs has been described (Bulloch *et al.* 1988); the SCPs were treated similarly.

Synthetic peptides

FMRFamide was purchased from Sigma, CRB or Peninsula Laboratories. FLRFamide came from Sigma and pQDPFLRFamide and SCP_B from Peninsula. SDPFLRFamide was synthesised, deprotected and purified by John Riehm (Ebberink *et al.* 1987); and NDPFLRFamide, NDPYLRFamide, SEPYLRFamide and SGYLAFPRMamide were synthesised by the Protein Chemistry Laboratory of the University of Florida Interdisciplinary Center for Biotechnology Research. SGYLAFPRMamide and NDPFLRFamide were removed from the resin and deprotected using the standard HF method, while the NDPYLRFamide and SEPYLRFamide were deprotected and removed from the resin by trifluoromethanesulphonic acid (TFMSA) according to the Applied Biosystems protocol. These four peptides were purified by HPLC and quantified by amino acid analysis. The amino acid compositions are shown in Table 2.

Bioassay

The method of Payza (1987) was used to bioassay peptides on ventricles isolated from *Helix aspersa*. After collection in the vicinity of Kingsbarns, Scotland, by Mr J. Brown, the snails were allowed to aestivate for several months. Ventricles were isolated and perfused for a minimum of 30 min with saline (composition in mmol l^{-1}: NaCl, 80; KCl, 5; $MgCl_2$, 5; $CaCl_2$, 7; Hepes, 20; pH 7.5) before any peptides were added. The perfusion rate was set at a minimum of 600 $\mu l\ min^{-1}$ and was then maintained at a constant level for a particular ventricle, although the rate varied slightly from one preparation to the next.

Only two peptides at a time, or three in the case of the heptapeptides, were tested on a particular ventricle; each pair or triplet was tested on a minimum of

Table 2. *Amino acid compositions of synthetic peptides*

Amino acid	SGYLAFPRM-amide	NDPFLRF-amide	NDPYLRF-amide	SEPYLRF-amide
Glycine	0.93		0.06	
Leucine	1.01	1.02	1.04	1.05
Proline	1.00	0.98	1.00	1.02
Arginine	1.00	1.00	1.00	1.00
Methionine	0.88		0.08	0.09
Alanine	0.98			
Phenylalanine	0.90	1.77	0.95	0.93
Serine	0.65		0.05	0.62
Tyrosine	0.99		0.98	1.03
Ammonia	0.96	1.35	1.70	1.57
Aspartic acid		1.90	2.01	
Glutamic acid			0.05	0.98

The absence of a numeral indicates less than 0.05 residue.

five ventricles, recorded either isometrically or isotonically. Roughly equipotent doses of each peptide in turn were applied 10 min apart. The percentage increase in tension or amplitude was plotted against the logarithm of the dose, and the potency ratio for the pair or triplet of agonists was estimated from the linear portion of the plots.

Results

Multiple peaks of FMRFamide-like immunoreactivity can be detected

Several peaks of FMRFamide-like immunoreactivity can be distinguished after HPLC fractionation of either whole snail or ganglionic extracts; the exact number depends on the antisera used for their characterisation. By using two antisera of differing specificity in parallel, we can distinguish at least five peaks in ganglionic extracts (Fig. 1A), of which only two, i.e. those containing FMRFamide and pQDPFLRFamide, had been completely identified in earlier work on *Helix*. We isolated them once again and, as expected, they contained the molecular ions for FMRFamide and pQDPFLRFamide (Table 3), but neither showed evidence of containing additional immunoreactive species.

Our analysis of three of the remaining immunoreactive peaks is presented below.

FLRFamide

One peak, at the elution position of FLRFamide, does not appear as a distinct peak with either RIA alone, but the intersection of the two immunoreactive profiles shows it clearly (Fig. 1A). This peptide is a minor component of the total immunoreactivity, so it tends to appear as a shoulder on the FMRFamide peak when assayed with most antisera, e.g. our S253. In contrast, our Q2 antiserum,

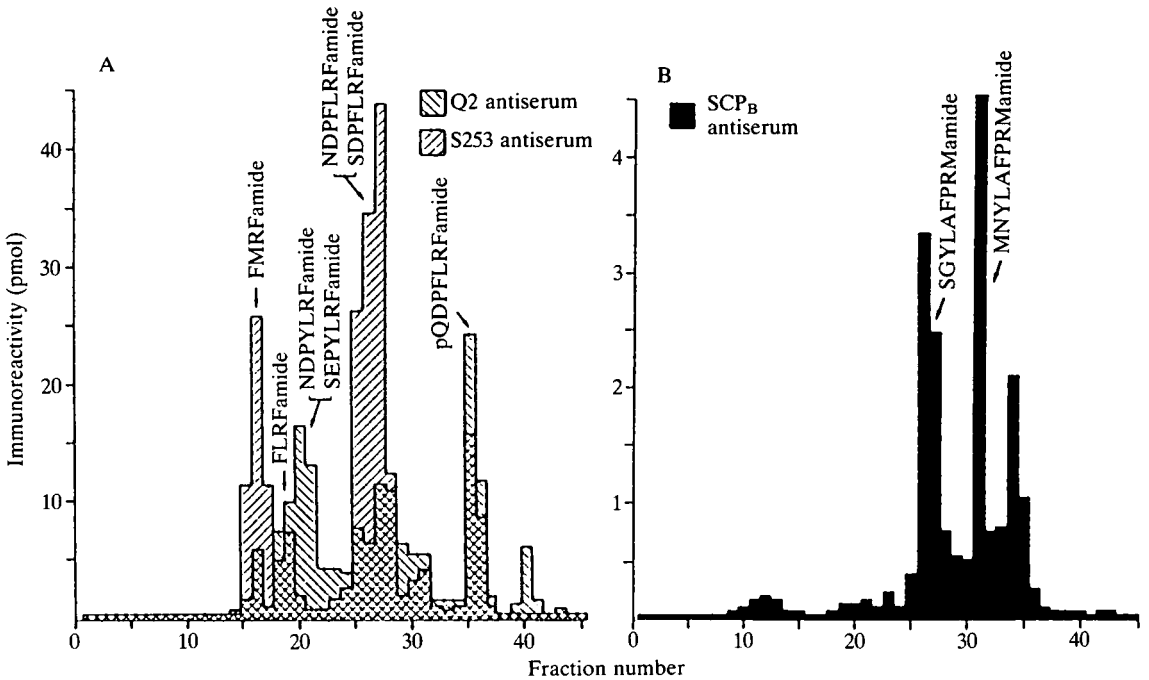

Fig. 1. Immunoreactivity profiles from an HPLC fractionation of a snail ganglion extract. After loading the extract (in 0.1 % aqueous trifluoroacetic acid, TFA) onto an RP-300 column (2.1 mm×220 mm), the flow was switched to 16 % acetonitrile (ACN) and a gradient (16–32 %, 0.1 % in TFA over 20 min) of ACN was started when the front reached the detector. 30 s fractions were collected and 2 μl samples of each were taken for three different radioimmunoassay (RIAs). (A) Immunoreactivity with two different RIAs for FMRFamide-related peptides. (B) Immunoreactivity with an RIA to SCP_B. The elution positions of relevant synthetic peptides are indicated.

which was raised to an extended FLRFamide, separates the FLRFamide and FMRFamide peaks, but merges FLRFamide with the following peak (Fig. 1A). FABms analysis of the putative FLRFamide peak showed a clear molecular ion at 581 (Table 3). Thus, its identity was confirmed.

SEPYLRFamide and NDPYLRFamide

The second unknown FaRP corresponds to a peak that had previously been reported to elute near FLRFamide and to contain a tyrosine for phenylalanine substitution (Price *et al.* 1987*b*). This peak is not very immunoreactive with the S253 antiserum, but reacts well with the Q2 antiserum (Fig. 1A). Our first batch, purified from an extract of 10 suboesophageal ganglia, was used for FABms analysis and showed a prominent ion at 923. This value is just 16 Da (one oxygen atom – the difference between Phe and Tyr) more than NDPFLRFamide (907 molecular ion), as expected from the amino acid composition we had reported earlier. From a somewhat larger batch (15–20 central nervous systems), we ended

Table 3. *Cardioexcitatory peptides of* Helix *identified by FABms*

Peptide	Calculated molecular ion†	Observed molecular ion*
FMRFamide	599.31	599.26
FLRFamide	581.36	581.39
SDPFLRFamide	880.47	880.54
NDPFLRFamide	907.48	907.58
pQDPFLRFamide	904.47	904.41
SEPYLRFamide	910.48	910.46
NDPYLRFamide	923.47	923.49
MNYLAFPRMamide‡	1173.55	1173.48
SGYLAFPRMamide‡	1056.53	1056.50

* Positively charged molecular ions were observed, so the observed weights correspond to the molecular weight plus 1 for the hydrogen.

† The molecular ions were calculated from the elemental composition and the weight of the most common isotope of each element.

‡ These peptides were identified after oxidation, so the calculated molecular ions were based on the methionines being the sulphoxides.

up with a peak that was obviously a doublet (Fig. 2A) as judged by its ultraviolet absorbance. Since both components of the doublet seemed immunoreactive, and the ratio of 280 to 210 nm absorbance was constant through the peak (Fig. 2A), we decided to analyse each of the two most immunoreactive fractions (17 and 18) separately. So we divided each in half – one half for sequencing and the other for FABms. From the earlier-eluting fraction we obtained only the sequence NDPYLRF (Fig. 2B) and a 923 molecular ion, confirming our identification of this component as NDPYLRFamide. The later-eluting immunoreactive fraction sequenced as expected for a mixture of two peptides which share a common C-terminal pentapeptide sequence (Fig. 2C). Since one is NDPYLRFamide, the other must be SEPYLRFamide, and both expected molecular ions occur in this fraction (923 and 910; Table 3), a further confirmation of this sequence assignment.

SDPFLRFamide and NDPFLRFamide

The last unidentified peak appeared at the common elution position of the three

Fig. 2. (A) Ultraviolet absorbance traces and immunoreactivity (Q2 antiserum) profile of the purified tyrosine-containing FaRPs. The active material was loaded onto an RP-300 column and eluted as described in Fig. 1. There is a lag of approximately 0.5 min between the ultraviolet detector and the fraction collector. The peaks are labelled with their elution times (in min) and the fractions taken for further analysis are labelled with their fraction numbers. (B,C) The level of each pertinent amino acid derivative is plotted *versus* sequencer cycle for sequencing runs of the two immunoreactive fractions spanning the peak shown in A. The early part of the peak (fraction 17) gave the sequence shown in B and the later part (fraction 18) gave the sequence shown in C.

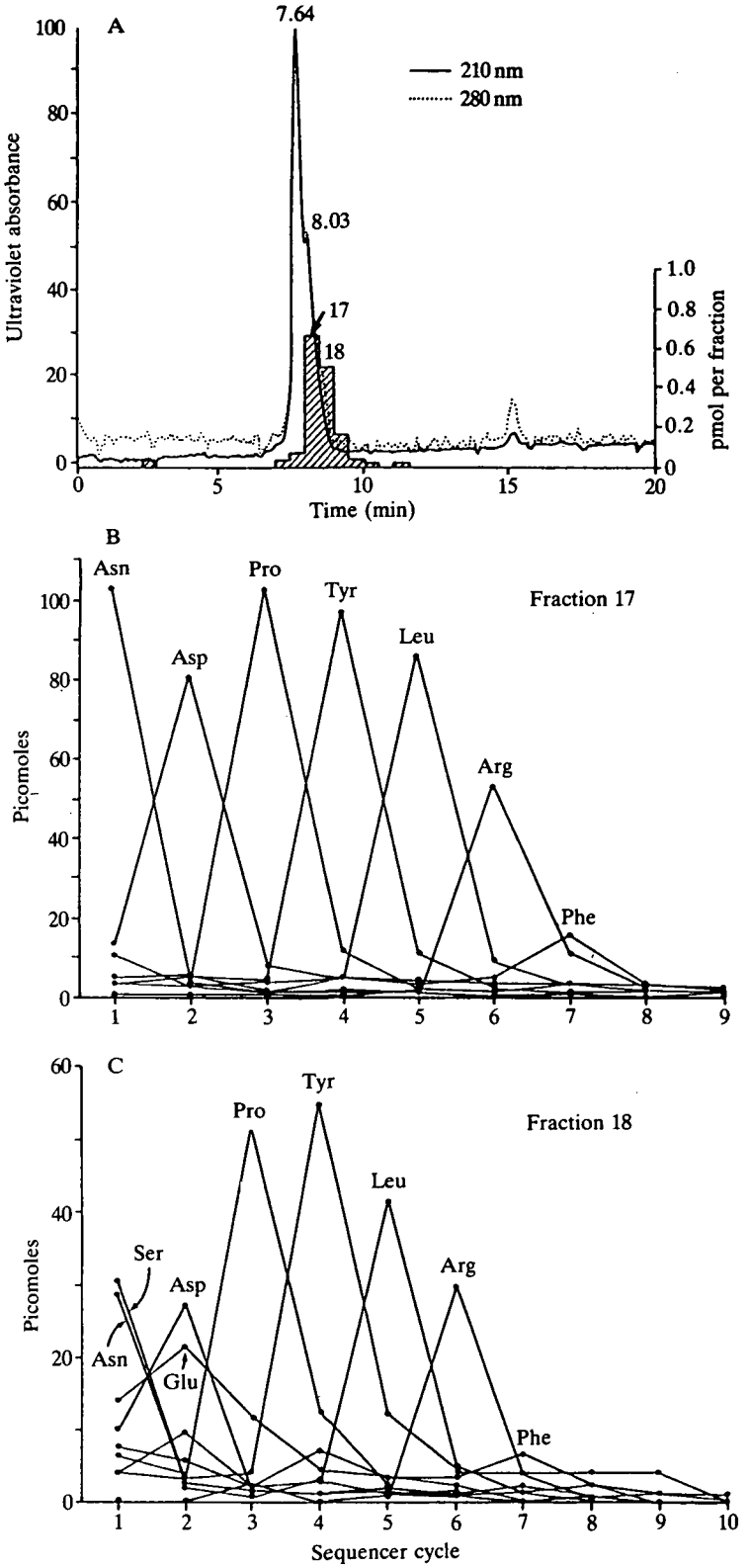
A
7.64
210 nm
280 nm
8.03
17
18
Ultraviolet absorbance
pmol per fraction
Time (min)
B
Asn
Pro
Tyr
Fraction 17
Leu
Asp
Arg
Phe
Picomoles
C
Tyr
Pro
Fraction 18
Leu
Ser
Asp
Arg
Asn
Glu
Phe
Picomoles
Sequencer cycle

Fig. 2

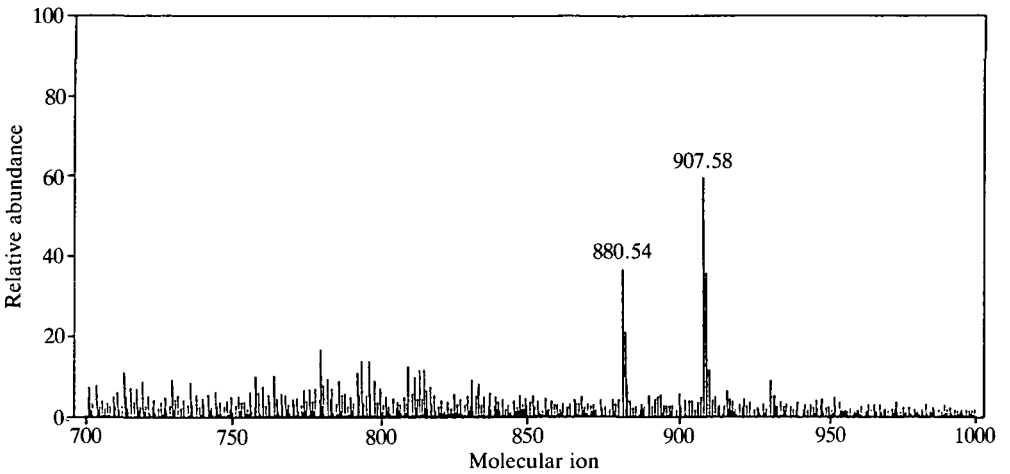

Fig. 3. FAB mass spectrum of the *Helix* HPLC peak which elutes at the common position of NDPFLRFamide, SDPFLRFamide and GDPFLRFamide. The two largest signals correspond to NDPFLRFamide (907.58) and SDPFLRFamide (880.54). There is no signal at 850 where GDPFLRFamide would appear.

peptides S-, N- and G-DPFLRFamide which are not separated by either of the solvent systems we used. All three had been identified previously in other pulmonate species (Price *et al.* 1987*a*; Ebberink *et al.* 1987), but not in *Helix*. FAB mass spectrometry of this purified peak clearly shows molecular ions corresponding to SDPFLRFamide at 880, and to NDPFLRFamide at 907 (Fig. 3; Table 3), but not to GDPFLRFamide which would appear at 850.

The SCPs

When extracts of only a few whole *Helix* or of dissected nervous systems were chromatographed, we observed three peaks of SCP immunoreactivity (Fig. 1B). Large batches of material (i.e. from 40 or more individuals) produced only two broader peaks. The peak eluting after the position expected for MNYLAFPRMamide (Fig. 1B) seems to be a form of MNYLAFPRMamide which reverts back to the normal form during further purification (data not shown). We think that it may be a complex of the SCP and a small molecule, but we do not have rigorous evidence for this.

MNYLAFPRMamide (SCP_B)

The later-eluting of the two largest SCP peaks is MNYLAFPRMamide. It elutes at the position expected for authentic SCP_B, it has the amino acid composition of SCP_B (Table 1), and the oxidised peptide has a molecular ion corresponding to SCP_B with two additional oxygens (Table 3).

SGYLAFPRMamide

The earliest SCP-like peak corresponds to a peptide reported previously, but

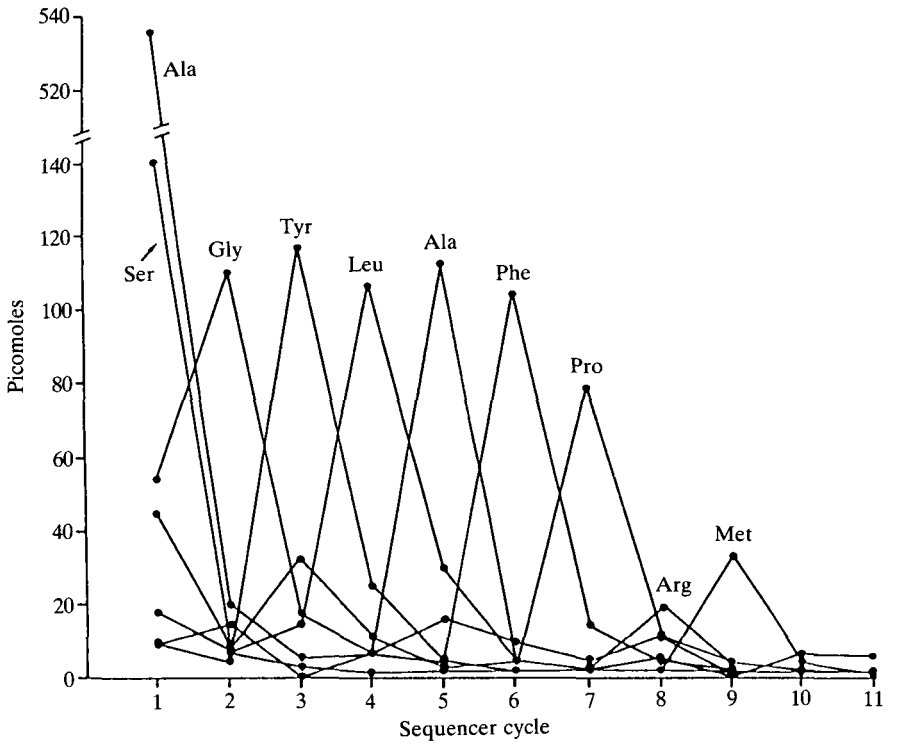

Fig. 4. Sequencing of the earlier-eluting SCP of *Helix*. The levels of pertinent amino acid phenylthiohydantoin (PTH) derivatives at each cycle are shown. The first cycle contained various amino acids in addition to those shown; the amounts of most were small enough to discount, but there were 260 pmol of Asn.

incompletely identified (Price, 1987). Analysis of this peak by FABms gives a molecular ion corresponding to that expected from its amino acid composition (Table 1) assuming the methionyl residue is the sulphoxide. Microsequencing of this peptide yielded X-Gly-Tyr-Leu-Ala-Phe-Pro-Arg-Met (Fig. 4), where the first position was ambiguous because of contamination with free amino acids. Serine is one of the predominant amino acids in the first sequencing cycle, and the only full sequence consistent with the observed molecular ion is Ser-Gly-Tyr-Leu-Ala-Phe-Pro-Arg-Met-amide (SGYLAFPRMamide). This peptide was synthesised and found to elute at the same time as the natural peptide.

Cardioexcitatory activity

All the peptides, both the FaRPs and the SCPs, affected the beat amplitude and tone of the ventricle more than the beat rate. Log dose–response curves were linear and approximately parallel in the middle of the concentration range, so potency comparisons are based on the peptide concentrations required to give equivalent responses in this range. Exemplary matches of equivalent responses of

pairs (or triplets) of the peptides on the same ventricle are shown (Figs 5 and 6), as are our quantitative estimates of potency (Fig. 7).

Equipotent doses of the tetrapeptide and heptapeptide FaRPs produce cardioexcitations that are indistinguishable (Fig. 5). Of the tetrapeptides, FMRFamide is about 10 times less potent than FLRFamide (Fig. 5A). The five heptapeptides were approximately equipotent (Fig. 5D–F), about 10 times more potent than FLRFamide (Fig. 5B), and about 100 times more potent than FMRFamide (Fig. 5C).

The heptapeptides and nonapeptides all had inhibitory effects on isolated *Helix* ventricles, particularly towards the high end of their concentration ranges. But these effects were idiosyncratic, occurring more frequently with some peptides, and on some ventricles, than others. Even when inhibiting the beat, however, the heptapeptide FaRPs were approximately equipotent (Fig. 5E).

The two SCPs of *Helix*, MNYLAFPRMamide and SGYLAFPRMamide, also gave very similar responses, though SGYLAFPRMamide was consistently about three times more potent (Fig. 6A). Since the doses of these two peptides had been quantified by HPLC, this difference, though small, is probably reliable. The highest doses of the SCPs were inhibitory (Fig. 6D).

The differences in the effects of the SCPs and FaRPs were examined by comparing the actions of SGYLAFPRMamide with those of FLRFamide or pQDPFLRFamide on several ventricles (Fig. 6B–D). A response to an SCP was usually longer lasting than that to an equipotent dose of a FaRP, giving a broader peak (Fig. 6B–D). Moreover, the positive inotropic actions of the SCPs were often accompanied by a *decrease* in diastolic tone (e.g. Fig. 6A), an effect never seen with the FaRPs. SGYLAFPRMamide was approximately eight times more potent than pQDPFLRFamide and the other heptapeptide FaRPs (Fig. 6B), and sixty times more effective than FLRFamide (Fig. 6C).

Fig. 7 summarises the differences in potency between pairs of peptides.

Discussion

We have now identified nine endogenous peptides with excitatory effects on the *Helix* ventricle, and so some of the agents responsible for the cardioexcitatory activity of *Helix* ganglia are now more clearly delimited almost 30 years after their first detection. Although these nine peptides seem to account for the majority of the non-5-HT cardioexcitatory activity found in the ganglia, other minor stimulatory components remain to be discovered. The larger, and still unanswered, question is how the effects of these peptides are integrated to regulate cardiovascular function.

There is some evidence that the FMRFamide-related peptides can function as

Fig. 5. Responses of isolated *Helix* ventricles to approximately equipotent doses of pairs or triplets of FMRFamide peptides (isometric recordings). A standard volume (400 μl) of saline containing the concentration of peptide indicated below each dose was injected at the arrowhead.

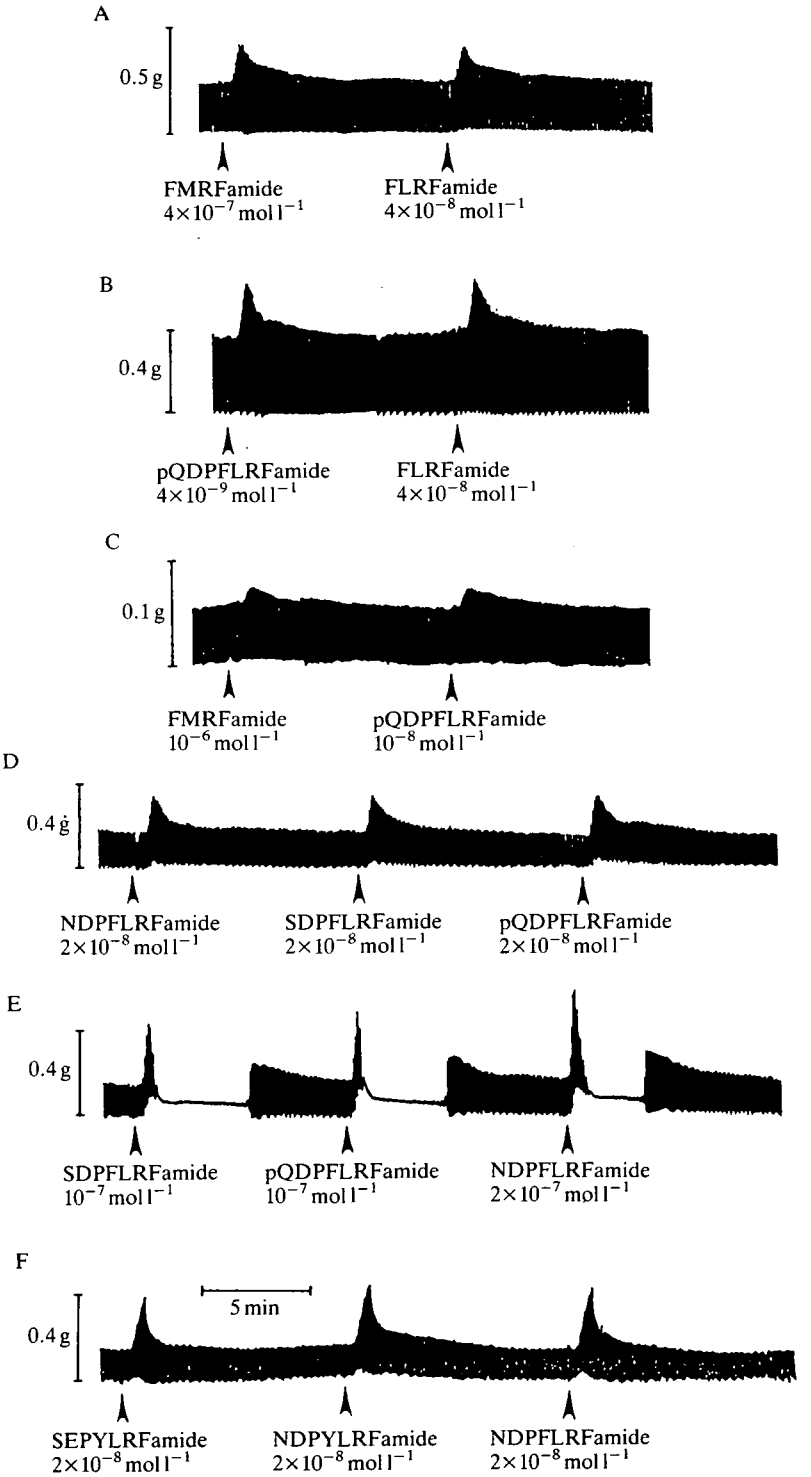
A
0.5 g
FMRFamide
4×10⁻⁷ mol l⁻¹
FLRFamide
4×10⁻⁸ mol l⁻¹
B
0.4 g
pQDPFLRFamide
4×10⁻⁹ mol l⁻¹
FLRFamide
4×10⁻⁸ mol l⁻¹
C
0.1 g
FMRFamide
10⁻⁶ mol l⁻¹
pQDPFLRFamide
10⁻⁸ mol l⁻¹
D
0.4 g
NDPFLRFamide
2×10⁻⁸ mol l⁻¹
SDPFLRFamide
2×10⁻⁸ mol l⁻¹
pQDPFLRFamide
2×10⁻⁸ mol l⁻¹
E
0.4 g
SDPFLRFamide
10⁻⁷ mol l⁻¹
pQDPFLRFamide
10⁻⁷ mol l⁻¹
NDPFLRFamide
2×10⁻⁷ mol l⁻¹
F
5 min
0.4 g
SEPYLRFamide
2×10⁻⁸ mol l⁻¹
NDPYLRFamide
2×10⁻⁸ mol l⁻¹
NDPFLRFamide
2×10⁻⁸ mol l⁻¹

Fig. 5

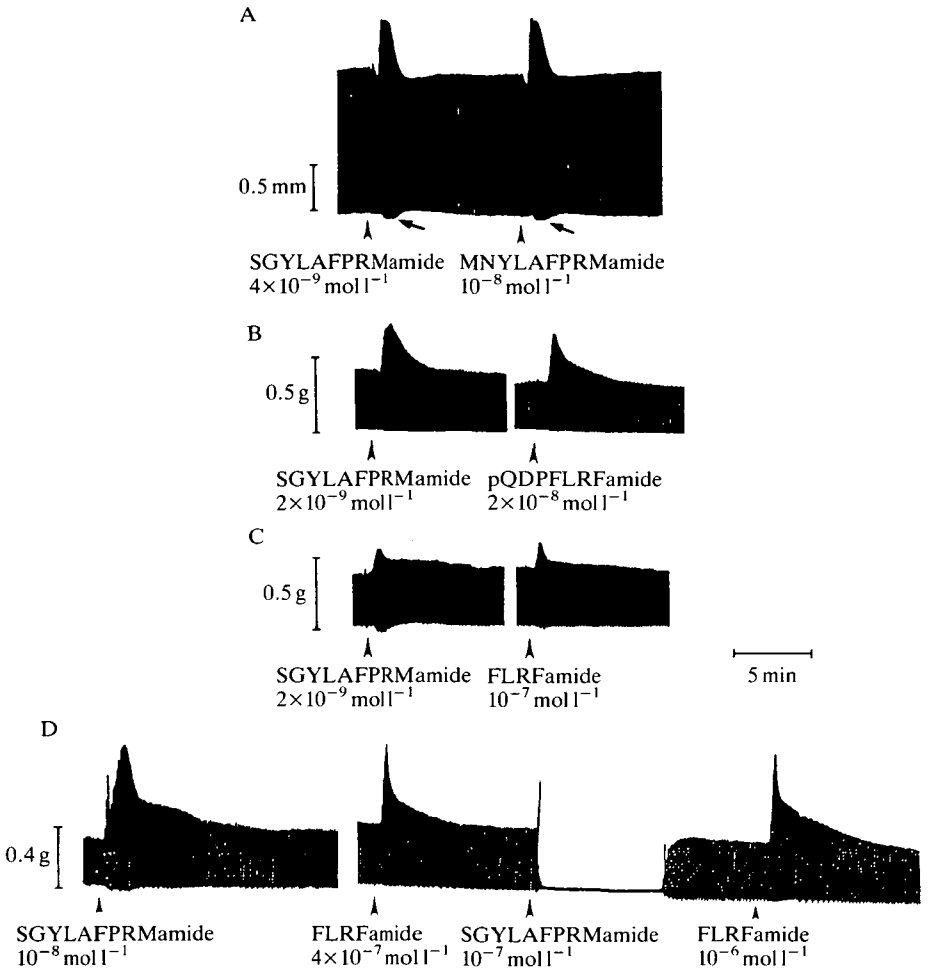

Fig. 6. Responses of isolated *Helix* ventricles to approximately equipotent doses of SCPs or FaRPs. A decrease in the diastolic tone (arrows) was often seen in response to the SCPs. A standard volume (400 μl) of saline containing the concentration of peptide indicated below each dose was injected at the arrowhead. The break in the trace indicates that the doses were not consecutive, though they are shown in the order of application. (A) Isotonic recording; (B–D) isometric recordings.

both cardioregulatory hormones and transmitters: immunoreactive FMRFamide is present in *Helix* blood (Price *et al.* 1985), and also in the heart itself (Lehman and Price, 1987). In contrast, no SCP bioactivity could be found in the blood or heart of either *Helix* (Lloyd, 1978) or *Aplysia* (Lloyd *et al.* 1985). Nevertheless, the SCPs are the most potent cardioexcitors of the nine, so they might still influence cardioregulation at levels not detectable by gel chromatography and bioassay.

The structures of the two SCPs we have isolated from *Helix* are compatible with an SCP precursor very similar to that found in *Aplysia* (Mahon *et al.* 1985). Since

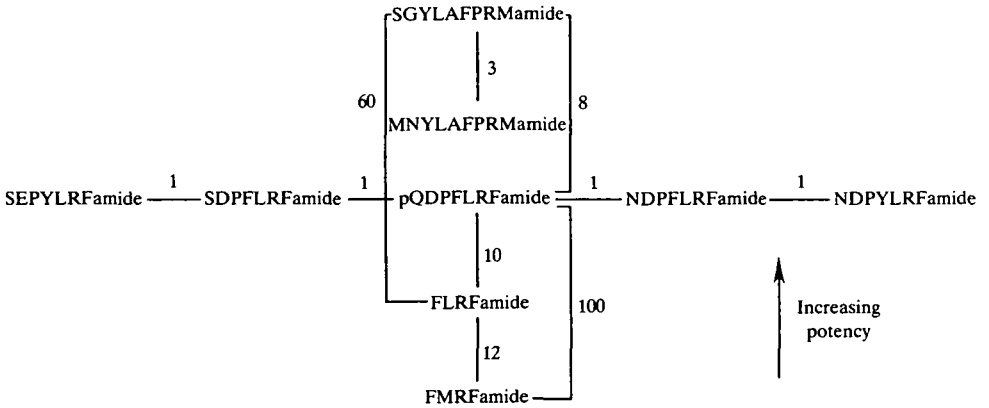

Fig. 7. The relative potencies of the nine known SCP- and FMRFamide-related peptides on the isolated ventricle from aestivating animals. The order of potency decreases from the top to the bottom. The numbers indicate the relative potencies.

one SCP of *Helix* is identical to SCP_B of *Aplysia*, the other would seem to be the *Helix* counterpart of SCP_A. Indeed, the replacement of a single cytosine with a thymidine in the *Aplysia* SCP precursor gene would change the proline in the third position of SCP_A to a serine, and the resultant precursor would be processed to the novel SCP reported here.

Earlier work on the FMRFamide-related heptapeptides of the pulmonates indicated that each species has two different heptapeptides (Ebberink *et al.* 1987; Price *et al.* 1987*a,b*), and we expected *Helix* to have only two: pQDPFLRFamide and one other. In retrospect, our previous work on *Helix* had revealed a mixture of NDPFLRFamide and SDPFLRFamide (see amino acid composition in Price *et al.* 1987*b*), but we could not interpret it as such, because serine, glycine and aspartic acid are common free amino acid contaminants. The use of FABms, as in this study, reduces the problem created by free amino acid contaminants.

Price *et al.* (1987*b*) hypothesised that two heptapeptides are encoded by a gene derived from the 5′ end of the FMRFamide precursor. This speculation is still untested but it cannot be entirely accurate. Instead, either two heptapeptide genes occur in *Helix*, or a single heptapeptide precursor must contain at least five heptapeptides. However, our further speculation – that one or more heptapeptide precursors originated from the *N*-terminal region of the FMRFamide precursor – is strengthened by the discovery of two heptapeptides ending -YLRFamide; i.e. the *N*-terminal region of the *Aplysia* precursor does encode such a peptide.

The work reported here illustrates the utility of FABms in the identification of peptides. Though the three heptapeptides (N-, S- and G-DPFLRFamide) cannot be separated with acidic solvent systems on several C-18 columns, we can continue to use these otherwise desirable acidic systems if we identify the peptides that are present with FABms. In addition, the FABms identification of the molecular ion provides a more positive test of identity than does HPLC co-elution.

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