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The Peptide pQFYRFamide Is Encoded on the FMRFamide Precursor of the Snail *Helix aspersa* but Does Not Activate the FMRFamide-Gated Sodium Current

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Abstract. The complete sequence of the FMRFamide precursor cDNA from *Helix aspersa* is reported here. Since the 5′ end of this cDNA is identical to that of the precursor that encodes the heptapeptide analogs of FLRFamide, the two transcripts are probably derived by alternative RNA splicing. A novel pentapeptide, Glp-Phe-Tyr-Arg-Phe-NH_2 (pQFYRFamide), predicted from the cDNA sequence, was purified from extracts of *H. aspersa* ganglia and identified by mass spectroscopy. Partial gene sequences for the FMRFamide precursors of two closely related pulmonate species—*Cepaea nemoralis* and *Polydontes acutangula*—were also determined and compared with the cDNA sequence of *H. aspersa* and a partial gene sequence previously determined from *H. pomatia*. Not only are the FMRFamide-related sequences and proteolytic processing sites conserved, but the linear arrangement of these landmarks is also retained. Synthetic pQFYRFamide has some effects on the isolated heart and on neuronal potassium currents in *H. aspersa* that are similar to those of FMRFamide, but it does not activate the neuronal FMRFamide-gated sodium channel.

Introduction

The tetrapeptide FMRFamide (Price and Greenberg, 1977) is undoubtedly present in all molluscs (Greenberg and Price, 1992), but the mRNA encoding the FMRFamide precursor has only been completely described from an opisthobranch, *Aplysia californica* (Schaefer *et al.*, 1985; Taussig and Scheller, 1986), and a pulmonate, *Lymnaea stagnalis* (Linacre *et al.*, 1990). Although only a few stretches of amino acid sequence are common to the precursors of these two species [*i.e.*, a single copy of FLRFamide, a sequence SEEPLYRKRRS which includes a tetrabasic proteolytic processing site, and the multiple copies of FMRFamide], the general organization of these two precursors is similar (see Greenberg and Price, 1992).

Pulmonate snails, but not other molluscs, also contain heptapeptides of the form XDPFLRFamide (X = N, S, G, pQ) (Price *et al.*, 1990). These heptapeptide analogs of FLRFamide are encoded on a different precursor from FMRFamide (Saunders *et al.*, 1991; Lutz *et al.*, 1992) and are expressed in different neuronal populations (Bright *et al.*, 1993; Cottrell *et al.*, 1992) in both *Lymnaea stagnalis* and *Helix aspersa*. In *L. stagnalis*, the two precursors are alternatively spliced variants of a single gene which share the same first exon (Saunders *et al.*, 1992). Whether a similar alternative splicing mechanism occurs in *H. aspersa* is unclear because only a partial cDNA encoding the tetrapeptides had been previously isolated (Lutz *et al.*, 1992), notwithstanding that the cDNA encoding the heptapeptide precursor in *H. aspersa* has been fully sequenced (Cottrell *et al.*, 1994).

Because conservation of structure is often associated with functionally important regions of a protein, and because the FMRFamide precursor from only one other pulmonate is fully known, we have determined the complete sequence of the FMRFamide precursor in *H.*

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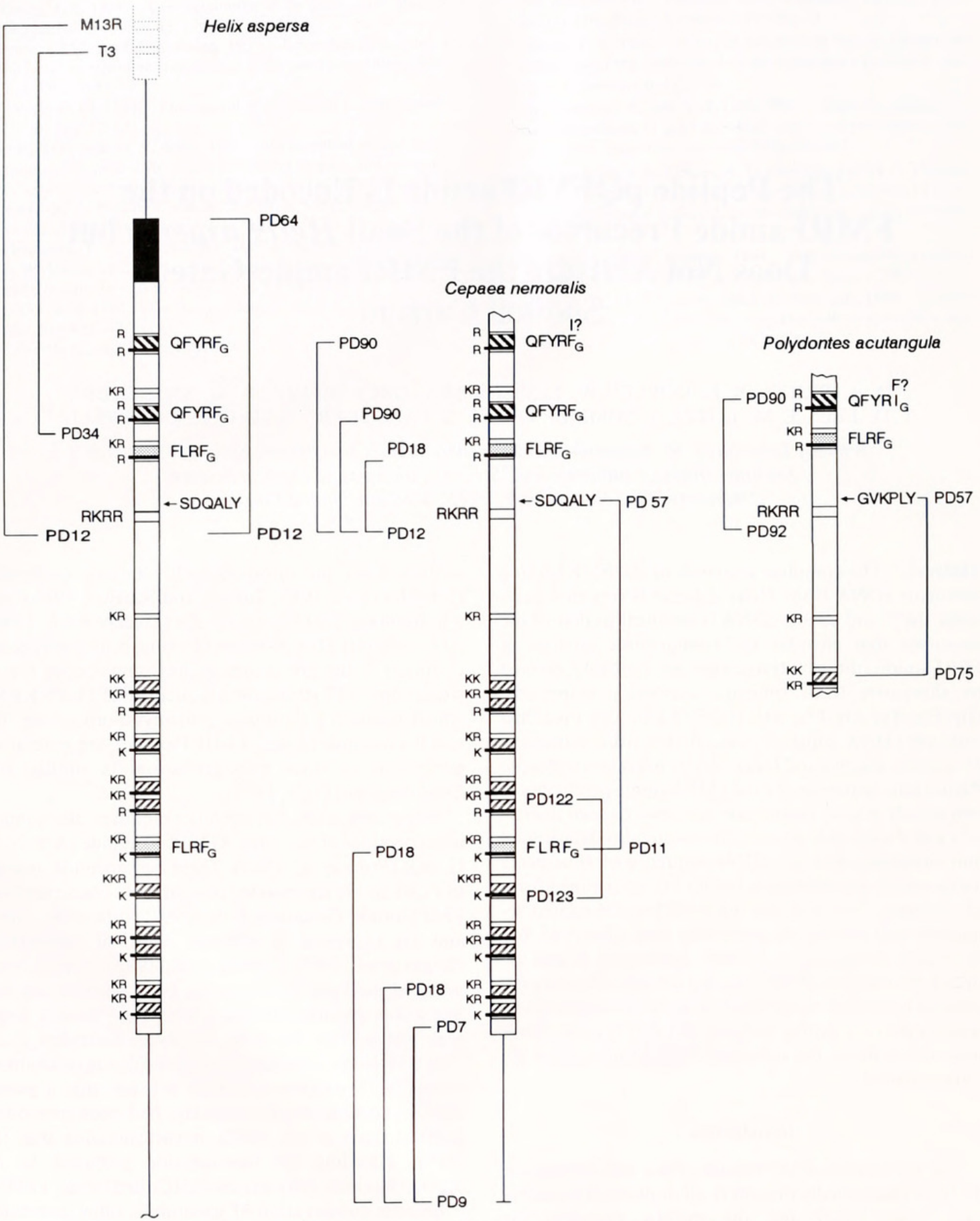


Figure 1. Composite schematic representation of the complete FMRFamide precursor of *Helix aspersa* (GenBank L20768) and precursor fragments from *Cepaea nemoralis* (GenBank U02488) and *Polydontes acutangula*. The noncoding regions are shown as vertical lines and the coding regions as columns. The columns are positioned so that the tetrabasic sequences (RKRR) common to all are horizontally aligned.

aspersa, showing in the process that alternative splicing occurs in this species as well as in *L. stagnalis*. We have also broadened the comparison by examining two additional pulmonate snail species and have established, thereby, landmark sequences that seem to be common features of the FMRFamide precursors of pulmonate and opisthobranch molluscs.

The resulting sequences have revealed a putative novel peptide (pQFYRFamide) that has now been purified from extracts. The pharmacology of FMRFamide-related peptides has already been studied extensively in *H. aspersa*, and we know that the heptapeptide analogs of FLRFamide have some biological actions on peripheral organs and neurons that are distinct from those of FMRFamide and FLRFamide (Lehman and Greenberg, 1987; Cottrell and Davies, 1987). We have therefore surveyed the pharmacology of synthetic pQFYRFamide and report here that, although this pentapeptide has some of the same actions as FMRFamide on hearts and neurons of *H. aspersa*, it has no effect on the neuronal sodium channel that is directly gated by FMRFamide (Cottrell *et al.*, 1990; Green *et al.*, 1994).

Materials and Methods

Animals

Three species of snails from the pulmonate order Stylommatophora, superfamily Helicoidae, were used in this study. Two species, *Helix aspersa* and *Cepaea nemoralis*, are members of the family Helicidae and the third, *Polydontes acutangula*, is in the family Camaenidae. Note that *Lymnaea stagnalis*, another frequently studied pulmonate, is in the order Basommatophora.

Those specimens of *H. aspersa* used in the purification of pQFYRFamide and in the pharmacological testing of that peptide on snail hearts were collected in Fullerton, California, and shipped to St. Augustine, Florida, by Robert A. Koch. The cDNA library was made from *H. aspersa* collected in St. Andrews, Scotland, and these animals were also used in electrophysiological experiments. Specimens of *Cepaea* were collected in Bellmore, New York, whereas *Polydontes* were obtained in El Yunque rain forest in Puerto Rico.

Library construction

RNA was isolated from clusters of neurons located in the parietal ganglia of *H. aspersa*. These clusters consist almost entirely of neurons immunoreactive to FMRFamide peptides (Cottrell *et al.*, 1992). A directional cDNA library was constructed using the Lambda-Zap kit (Stratagene).

PCR amplifications

Preparation of template DNA. Aliquots (typically 5 μl, but always less than 10 μl) of the amplified cDNA library were used directly in the PCR. For amplification of genomic DNA from *Cepaea* or *Polydontes*, a small piece of tissue (typically 1 ganglionic ring, 10 mg) was heated for 10 min at 95°C in about a 5-fold excess of 100 m*M* NaOH (50 μl for a ganglion) and was then centrifuged for 5 min at maximum speed in an Eppendorf centrifuge. The supernatant was transferred to a clean tube and diluted 10-fold with water. Aliquots (0.3 to 5 μl) were used for PCR.

Standard amplification. All of the PCR components, except the DNA polymerase, were assembled in a total volume of 80 μl in a 0.5-ml tube and overlaid with mineral oil. The tube was heated to 95°C for 10 min, cooled to 80°C, the DNA polymerase (Taq, Promega, 2.5 U in 20 μl) added through the oil, and the PCR cycles (Perkin Elmer Cetus thermal cycler) started (30 to 40 cycles of 1 min at 94°C, 1 min at 50°C, 2 min at 72°C). The PCR reaction mixture contained buffer (Promega: 50 m*M* KCl, 10 m*M* Tris-HCl, 0.1% Triton X-100, pH 9.0 at 25°C), 1.5 m*M* Mg, 200 μ*M* of each dNTP (Pharmacia), template and primers (20 to 200 μmol). An aliquot (10 μl) was run on an agarose gel, and reactions that showed products of the expected size were purified for cloning as follows. First, the aqueous phase containing the PCR product was removed from under the oil layer and rolled around on a sheet of Parafilm to remove residual oil droplets (Whitehouse and Spears, 1991). The DNA was then precipitated by the addition of an equal volume of 5 *M* ammonium acetate followed by two volumes of isopropanol (storage overnight at −20°C). The precipitate was then washed with 70% ethanol, dried in a Speed-Vac, and resuspended in 10 μl water. Aliquots (1–7 μl) were

The six amino acid residues just upstream of the tetrabasic sites are also labeled. The positions of individual peptides are stippled or hatched; the copies of FMRFamide are not labeled. Putative, amide-donating, glycine residues are shown as dark lines extending to the left of the column. Thin cross-lines delimit the boundaries of putative, basic processing sites. The signal sequence of the *H. aspersa* precursor is shown in solid black. The segments that were amplified by PCR, then cloned and sequenced, are indicated by brackets labeled with the primers used. Alternative amino acid residues encoded by degenerate PCR primers are shown above the most likely sequence and are followed by question marks. The vector region expected to adjoin the cloned *H. aspersa* sequence is shown with dotted lines. The *H. aspersa* 3′ noncoding region (see Lutz *et al.*, 1992) extends further than shown (indicated by a tilde).

ligated into the pGEM-T vector (Promega), which was then transformed into *E. coli* (JM109). Colonies were analyzed by PCR with primers taken from either side of the cloning site. Clones containing an insert of the appropriate size were sequenced with Sequenase (US Biochemicals, Cleveland, Ohio) kits.

Nested PCR. For the initial amplification, 5 μl from the library was used as the template for a pair of primers. [For an example, consider M13R and PD12; see Fig. 1 for location.] From the initial reaction product, 0.1% to 1% was used as template for the secondary amplifications with two additional primers chosen from the targeted PCR product, and only 25 cycles of amplification were done. [Continuing the example, see T3 and PD34 in Fig. 1.]

Primers. The following oligonucleotide primers were synthesized by the DNA synthesis facility of the University of Florida Interdisciplinary Center for Biotechnology Research. Seven of these primer sequences were taken directly from known DNA sequences: PD7, PD9, and PD12 are from the FMRFamide-encoding clone HF1 (Lutz *et al.*, 1992); PD64 is from the heptapeptide-encoding clone HF4 (also Lutz *et al.*, 1992); M13 rev and T3 promoter are from near the insertion site of the plasmid cloning vector Bluescript (Stratagene); and PD92 is from the first FMRFamide-encoding PCR product obtained from *Polydontes*. The remaining eight primers were designed from amino acid sequences rather than DNA sequences and are degenerate; the mixed bases are parenthetical in the following list. Inosine (I) was used in positions where all four bases could encode the desired amino acid. Some primers to repeated sequences were designed to be only partially degenerate so that they would *not* match every repeat exactly. For example, PD11, an antisense primer to some DNA sequences encoding RFMRFGK (R or S) exactly matches only one repeat in the *Cepaea* FMRFamide gene. The primer binding sites for all of these oligonucleotides are shown in Figure 1.

PD7 (sense): CGTGGGGATTCAGAAACATCATGA
PD9 (anti-sense): TAGCGCTAGAACCCTACTACCGAT
PD11 (anti-sense): CTTTTCCC(AG)AA(TC)CTCAT(GA)AA(TC)CG
PD12 (anti-sense): AGATTGTTGGTTAGCGTCAGCTGA
PD18 (sense): GAIAAG(AC)GITTC(TC)TG(AC)GGTTCGG
PD34 (anti-sense): A(AG)(AG)AAIC(GT)(CT)TT(AG)TC(TC)TG(AG)TAIGG
PD57 (sense): TCAGA(GC)(GC)AG(GC)CI(CT)TITA(TC)(AC)GIAA(AG)(AC)G
PD64 (sense): ACTAGTCTGTGCCTCACCATC
PD75 (anti-sense): TTCCC(AG)AA(CT)CTCAT(AG)AAIC(GT)(CT)TT
PD90 (sense): (CA)G(GA)CAGTT(TC)TA(TC)CGA(TA)T(TC)GG(CT)(CA)G
PD92 (anti-sense): CTCGGCTGTTTGGTTGGCACT
PD122 (sense): TT(TC)ATG(CA)GITT(TC)GGIAG(AG)GGCGA
PD123 (anti-sense): CTT(CG)CCGAACCTCATGAATCTTTT
M13 rev (NEB # 1233): AGCGGATAACAATTTCACACAGGA
T3 promoter (NEB # 1228): ATTAACCCTCACTAAAGGGA

Peptide extraction

Ganglia were added to acetone to give a final concentration of 2 g/10 ml, and left at −20°C until processed further, but at least overnight. The acetone was decanted from the tissue, centrifuged, and the supernatant filtered through plain nylon (0.45-μm pore size; MSI, Westboro, Massachusetts). The clarified extract was reduced in volume to about 10% on a rotary evaporator with heating to 50°C, leaving a primarily aqueous phase that was again clarified by filtration.

HPLC fractionation

In all cases, the samples were pumped directly onto the reverse phase column (2.1 × 200 mm, Brownlee Aquapore Octyl) through the aqueous solvent pump. The absorbance of the effluent was monitored at two wavelengths (214 and 280 nm), and fractions (about 0.25 ml) were collected every 30 s. The flow rate was 0.5 ml/min throughout. Fractions to be rerun for further purification were pooled and diluted several-fold with the new aqueous solvent before being loaded onto the column. Three solvent systems were used: 0.1% trifluoroacetic acid (TFA) in water/0.1% TFA in 80% acetonitrile; the same with heptafluorobutyric acid (HFBA) substituted for TFA; and 5 m*M* sodium phosphate (pH 7.0) in water/5 m*M* sodium phosphate in 60% acetonitrile.

Radioimmunoassay

Radioimmunoassay, with either of two antisera (S253 or Q2), was used to analyze the fractions as previously described (Lesser and Greenberg, 1993).

Peptide synthesis

The peptide pQFYRFamide was custom synthesized by Research Genetics (Huntsville, Alabama). Other peptides were synthesized by the peptide synthesis laboratory of the University of Florida Interdisciplinary Center for Biotechnology Research or purchased from Sigma Chemical Co.

Liquid chromatography–mass spectrometry (LC–MS)

Mass spectra were recorded in the positive ion mode on a TSQ-700 triple quadrapole instrument (Finnigan-MAT, San Jose, California) equipped with an electrospray ion source operating at atmospheric pressure. The

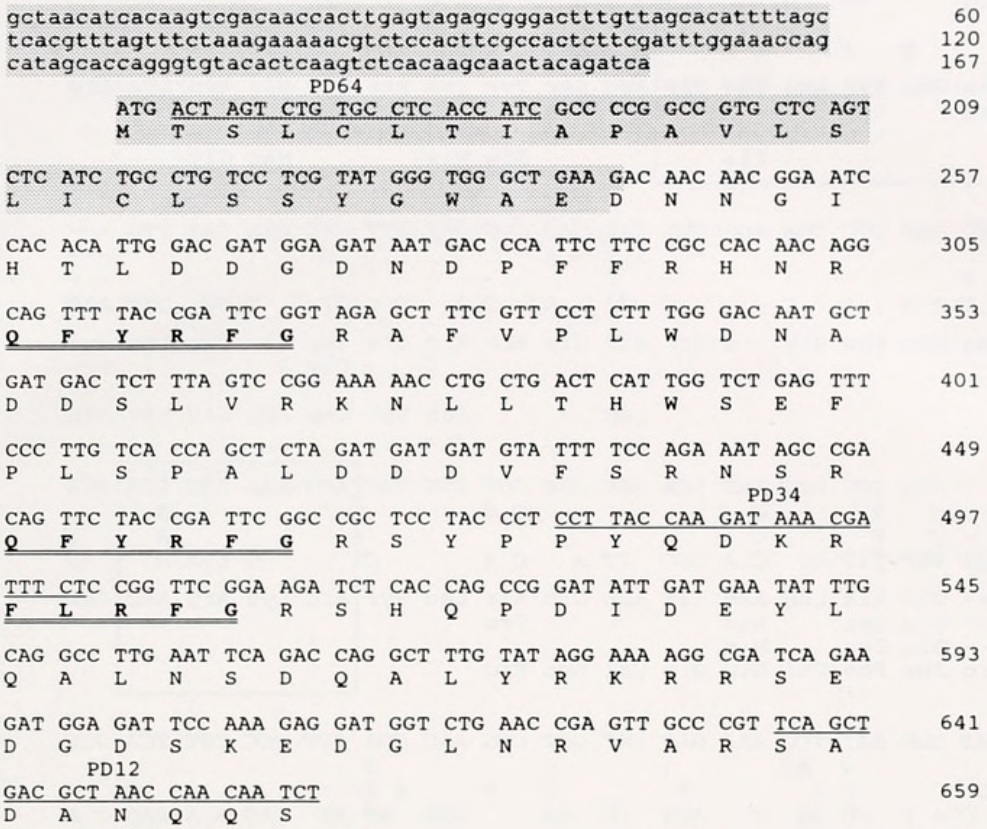


Figure 2. The nucleotide and deduced amino acid sequences of the 5′ end of the FMRFamide precursor from *Helix aspersa.* The binding sites for the indicated primers (PD64, PD34, and PD12) are underlined. The portion of the sequence that is identical to the heptapeptide precursor is shaded, and the amino acids of the mature peptides (including the amide donor, glycine) are double underlined.

electrospray needle was operated at a voltage differential of 3–4 kV, and a sheath flow of 2 μl/min of methoxymethanol was used. Scans were acquired every 3 s (Swiderek *et al.,* 1996).

The chromatography was performed on a microcapillary high-performance liquid chromatography (HPLC) system built at the Beckman Research Institute, City of Hope (Davis *et al.,* 1994, 1995). Fused silica columns with an inner diameter of 250 μm were packed with Vydac 3-μm C18 RP support. The sample was eluted with a gradient from 98% solvent A (0.11% TFA) to 62% solvent B (90% acetonitrile, 0.07% TFA) over 30 min with a flow of 2 μl/min. The UV absorbance (200 nm) was monitored before the sample was introduced into the mass spectrometer. Spectra were generated by averaging scans containing the peak, and the masses were calculated with data reduction software (Finnigan MAT BIOMASS). The determined mass values of the molecular ions are accurate to within a few tenths of a dalton, reported values are rounded down to the nearest integral value.

Biological assays

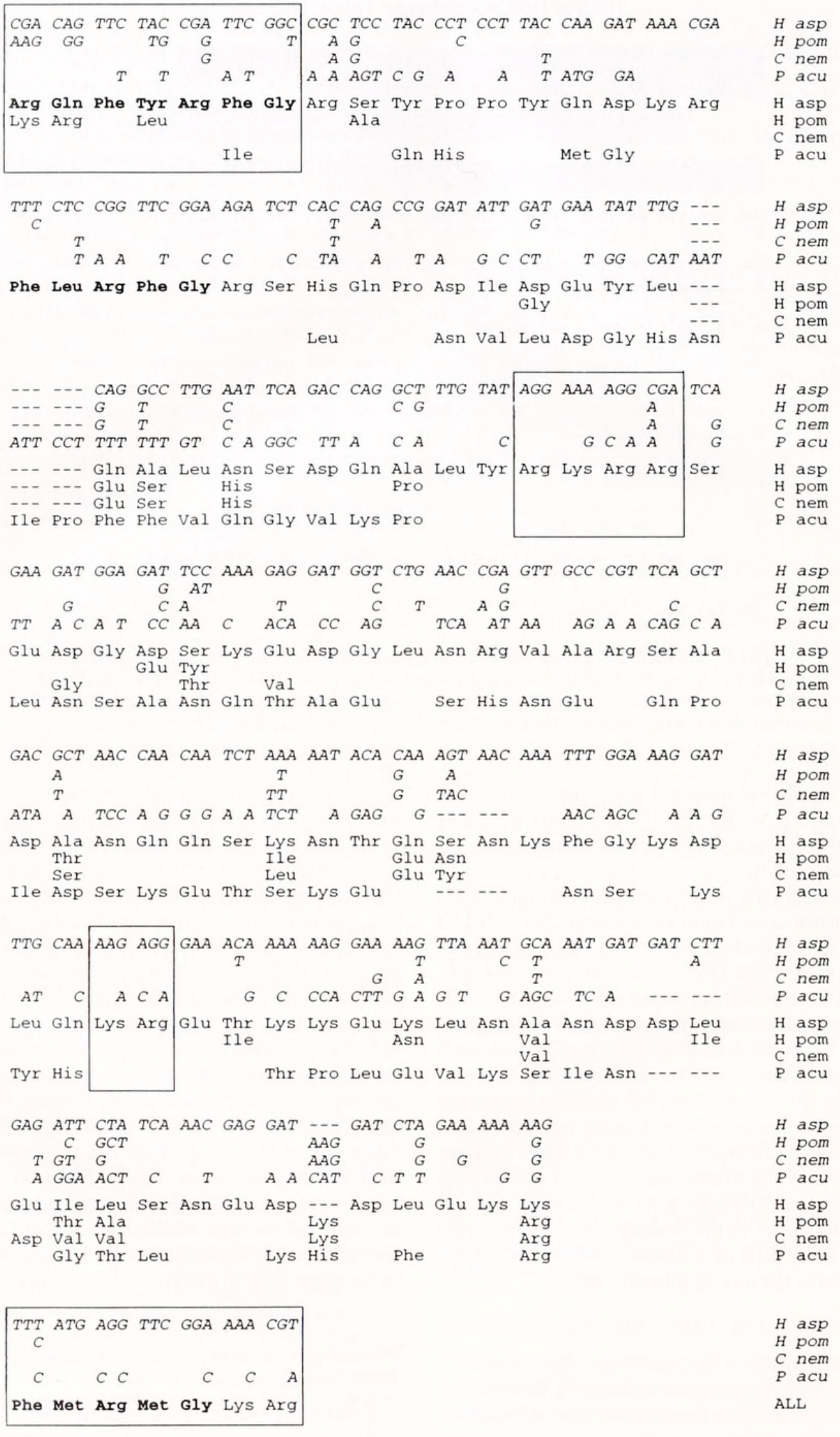
The isolated heart. The perfused ventricle of *H. aspersa* was used as described previously (Lesser and Greenberg, 1993) to assay the cardioactivity of synthetic pQFYRFamide.

Recording from identified neurons. Electrical recordings were made from two identified neurons (C1 and C2) in the cerebral ganglia of *H. aspersa;* neither neuron is a known follower of a FMRFamide-containing cell (see Cottrell and Davies, 1987). The physiological solution had the following composition (m*M*): NaCl (90), KCl (5), $MgCl_2$ (5), $CaCl_2$ (7), HEPES (20), and the pH was adjusted to 7.4 with NaOH. Neurons were voltage clamped with a Dagan 8100 single electrode clamp system with low-resistance micropipettes containing 200 m*M* KCl. Peptide solutions (100 μ*M*) were prepared in an appropriate physiological medium and were usually pressure applied (Picospritzer II, General Valve Corporation). However, a barium-containing physiological solution (10 m*M* KCl, 25 m*M* $BaCl_2$, 5 m*M* $MgCl_2$, 75 m*M* tetraethylammonium chloride, 3 m*M* 4-amino pyridine, 5 m*M* Tris HCl, pH 7.5) was used during the measurement of currents passing through calcium channels, and the peptide was added to the bathing solution prior to the generation of a current-voltage curve.

Results

The complete sequence of the mRNA encoding the FMRFamide precursor

A partial sequence had previously been isolated from a *Helix aspersa* cDNA library; it extended from an


CGA CAG TTC TAC CGA TTC GGC CGC TCC TAC CCT CCT TAC CAA GAT AAA CGA H asp
AAG GG TG G T A G C H pom
G A G T C nem
T T A T A A AGT C G A A T ATG GA P acu
Arg Gln Phe Tyr Arg Phe Gly Arg Ser Tyr Pro Pro Tyr Gln Asp Lys Arg H asp
Lys Arg Leu Ala H pom
C nem
Ile Gln His Met Gly P acu
TTT CTC CGG TTC GGA AGA TCT CAC CAG CCG GAT ATT GAT GAA TAT TTG --- H asp
C T A G --- H pom
T T --- C nem
T A A T C C C TA A T A G C CT T GG CAT AAT P acu
Phe Leu Arg Phe Gly Arg Ser His Gln Pro Asp Ile Asp Glu Tyr Leu --- H asp
Gly --- H pom
--- C nem
Leu Asn Val Leu Asp Gly His Asn P acu
--- --- CAG GCC TTG AAT TCA GAC CAG GCT TTG TAT AGG AAA AGG CGA TCA H asp
--- --- G T C C G A H pom
--- --- G T C A G C nem
ATT CCT TTT TTT GT C A GGC TT A C A C G C A A G P acu
--- --- Gln Ala Leu Asn Ser Asp Gln Ala Leu Tyr Arg Lys Arg Arg Ser H asp
--- --- Glu Ser His Pro H pom
--- --- Glu Ser His C nem
Ile Pro Phe Phe Val Gln Gly Val Lys Pro P acu
GAA GAT GGA GAT TCC AAA GAG GAT GGT CTG AAC CGA GTT GCC CGT TCA GCT H asp
G AT C G H pom
G C A T C T A G C nem
TT A C A T CC AA C ACA CC AG TCA AT AA AG A A CAG C A P acu
Glu Asp Gly Asp Ser Lys Glu Asp Gly Leu Asn Arg Val Ala Arg Ser Ala H asp
Glu Tyr H pom
Gly Thr Val C nem
Leu Asn Ser Ala Asn Gln Thr Ala Glu Ser His Asn Glu Gln Pro P acu
GAC GCT AAC CAA CAA TCT AAA AAT ACA CAA AGT AAC AAA TTT GGA AAG GAT H asp
A T G A H pom
T TT G TAC C nem
ATA A TCC A G G G A A TCT A GAG G --- --- AAC AGC A A G P acu
Asp Ala Asn Gln Gln Ser Lys Asn Thr Gln Ser Asn Lys Phe Gly Lys Asp H asp
Thr Ile Glu Asn H pom
Ser Leu Glu Tyr C nem
Ile Asp Ser Lys Glu Thr Ser Lys Glu --- --- Asn Ser Lys P acu
TTG CAA AAG AGG GAA ACA AAA AAG GAA AAG TTA AAT GCA AAT GAT GAT CTT H asp
T T C T A H pom
G A T C nem
AT C A C A G C CCA CTT G A G T G AGC TC A --- --- P acu
Leu Gln Lys Arg Glu Thr Lys Lys Glu Lys Leu Asn Ala Asn Asp Asp Leu H asp
Ile Asn Val Ile H pom
Val C nem
Tyr His Thr Pro Leu Glu Val Lys Ser Ile Asn --- --- P acu
GAG ATT CTA TCA AAC GAG GAT --- GAT CTA GAA AAA AAG H asp
C GCT AAG G G H pom
T GT G AAG G G G C nem
A GGA ACT C T A A CAT C T T G G P acu
Glu Ile Leu Ser Asn Glu Asp --- Asp Leu Glu Lys Lys H asp
Thr Ala Lys Arg H pom
Asp Val Val Lys Arg C nem
Gly Thr Leu Lys His Phe Arg P acu
TTT ATG AGG TTC GGA AAA CGT H asp
C H pom
C nem
C C C C A P acu
Phe Met Arg Met Gly Lys Arg ALL

EcoRI site 21 nucleotide residues upstream of the tetrabasic sequence (RKRR; indicated on Fig. 1), through the 3′ noncoding region (Lutz *et al.*, 1992). We have used the PCR-based strategy illustrated in Figure 1 to determine the sequence of the missing 5′ end of the cDNA. This additional sequence (shown in Fig. 2), taken together with that of Lutz *et al.* (1992), completes the sequence of the FMRFamide precursor of *H. aspersa*. The precursor is diagrammed in Figure 1, and the complete sequence has been deposited in GenBank, Accession L20768.

The 5′ end of the cDNA and the peptide precursor that it encodes [derived from these data and those of Lutz *et al.* (1992)] contain four noteworthy features (Fig. 1):

First, the 243 bases at the 5′ terminal (including the noncoding region and the signal sequence) are identical to those of the cDNA encoding the precursor of the heptapeptide analogs of FMRFamide (Lutz *et al.*, 1992). Therefore, the two transcripts (*i.e.*, those encoding the FMRFamide and heptapeptide precursors, respectively) must be derived by alternative RNA splicing, a finding also reported for *Lymnaea stagnalis* (reviewed by Benjamin and Burke, 1994).

Second, downstream from the signal sequence, the 5′ cDNA segment also encodes two copies of a previously unknown pentapeptide—pGlu-Phe-Tyr-Arg-Phe-NH_2 (pQFYRFamide)—as well as a copy of FLRFamide.

Third, 60 base pairs downstream from this FLRFamide, the cDNA encodes the tetrabasic cleavage site (Arg-Lys-Arg-Arg; RKRR) reported previously (Lutz *et al.*, 1992). The RKRR site also occurs in *Aplysia californica* (Taussig and Scheller, 1986) and *L. stagnalis* (Benjamin and Burke, 1994.)

Finally, 111 base pairs downstream, about $^3/_5$ of the distance between the tetrabasic (RKRR) site and the first copy of FMRFamide, the cDNA encodes a dibasic cleavage site (KR). This site is also found in *Aplysia californica* and *L. stagnalis* (Taussig and Scheller, 1986; Linacre *et al.*, 1990), but its relative position between the tetrabasic sequence and the first FMRFamide repeat varies.

In summary, the complete, derived precursor includes 10 copies of FMRFamide, 2 copies of FLRFamide (one in the midst of the FMRFamide repeats), 2 copies of the novel pentapeptide pQFYRFamide, and 2 conserved cleavage sites in addition to those flanking the FMRFamide-related peptides.

PCR amplification of other FMRFamide gene fragments

DNA from ganglia of the helicid snail *Cepaea nemoralis* and the camaenid snail *Polydontes acutangula* was prepared by crude alkaline lysis and amplified by PCR. In addition to the primers that had been employed for amplifications in *Helix*, we used another (PD92) that was synthesized to match exactly the *P. acutangula* sequence (see Fig. 1).

From *C. nemoralis* we obtained overlapping fragments covering much of the precursor, as shown in Figure 1 (for sequence see GenBank Accession U02488). The sequence of the *Cepaea* precursor is very similar to that of the two species of *Helix* (Figs. 1 and 3; Lutz *et al.*, 1992). Within the portion of the precursor that has now been sequenced from all three species, the *Cepaea* precursor has 88% nucleotide (nt) and 84% amino acid (aa) identity to *H. aspersa* and 90% nt and 81% aa identity to *H. pomatia*, and the two *Helix* species have 89% nt and 86% aa identity to each other.

The linear arrangement—in the precursors of *Cepaea* and *H. aspersa*—of the landmarks described above is virtually identical. In particular, the *Cepaea* precursor also encodes two copies of pQFYRFamide, a copy of FLRFamide, a tetrabasic site, and a dibasic cleavage site. Moreover, all of these features are in the same order and in virtually the same positions as in *H. aspersa;* the exception is that the distance between the dibasic cleavage site and the first copy of FMRFamide is one amino acid longer in *Cepaea* (Figs. 1 and 3). As expected, the segments between these landmarks are also very similar at both the amino acid and nucleotide levels (Fig. 3).

Although we have amplified only a small portion of the FMRFamide gene from *P. acutangula* (Fig. 1), most of the landmarks found in the cDNA from *H. aspersa* and *Cepaea* are present and recognizable. The 5′ end of the sequence encodes a copy of FLRFamide, the tetrabasic site, and the dibasic cleavage site. Upstream from the FLRFamide is the site where PCR primer PD90 annealed; this primer was designed to hybridize to DNA sequences encoding either pQFYRFamide or pQFYRIamide. Although the PCR product that was cloned and sequenced contains the code for Ile, we cannot be sure which amino acid is actually coded for in the native DNA.

In contrast to the landmarks themselves, the segments

Figure 3. A detailed comparison of the nucleotide and amino acid sequences from the mid-portion of the FMRFamide precursors of *Helix aspersa* (H asp), *Helix pomatia* (H pom, taken from Lutz *et al.*, 1992), *Cepaea nemoralis* (C nem), and *Polydontes acutangula* (P acu). The nucleotide sequences are shown in italics, and the mature FMRFamide-related peptides are shown in bold. The sequences were aligned by the introduction of gaps (– – –) to keep the boxed common features (pQFYRFamide, tetrabasic site, Lys-Arg, and first copy of FMRFamide) in register. The complete nucleotide and amino acid sequences are shown only for *H. aspersa*. For the other species, only the differences from *H. aspersa* are indicated, except for the gaps; the gaps are represented by dashes wherever they occur.

between them are poorly conserved in *P. acutangula* (Fig. 3). The precursor in this species is only 41% identical to *H. aspersa* at the nucleotide level, and 34% at the amino acid level. Moreover, although the overall length of the region between the two outside landmarks—pQFYR (I/F) amide and FMRFamide (see Fig. 1)—is exactly the same as for *H. aspersa,* three off-setting gaps were required to align the tetrabasic and dibasic cleavage sites in the two species. For another example, the peptide just 5′ of the tetrabasic site is especially dissimilar to that of *H. aspersa: i.e.,* it is longer (23 amino acids instead of 20) and, even with the gaps, only 17% conserved.

Isolation of pQFYRFamide

The predicted peptide pQFYRFamide was synthesized; it was as reactive as FMRFamide in an RIA with antiserum S253 which facilitated the purification. The synthetic peptide eluted at 12–13 min in the TFA/ACN solvent system, *i.e.,* coincident with a large peak of immunoreactivity observed with *H. aspersa* ganglion extracts (see Price *et al.,* 1990, for a typical pattern). Since this large peak also contains SDPFLRFamide and NDPFLRFamide, we sought a second solvent system that could separate pQFYRFamide from these two heptapeptides; the phosphate/ACN system proved to be satisfactory.

This fractionation procedure, with one modification, was applied separately to two *H. aspersa* extracts, one made from 50 pairs of cerebral ganglia and another from 30 subesophageal ganglia. The cerebral ganglion extract was given a preliminary fractionation with the HFBA/ACN system. Thereafter, each extract was fractionated on a TFA/ACN gradient, and the immunoreactive peaks from this run were further resolved with the phosphate/ACN solvent system. On this system, the cerebral ganglion extract showed a large peak at 19 min corresponding to synthetic pQFYRFamide, and a small peak at 16 min corresponding to the two heptapeptides (which are not separated in this system). The subesophageal ganglion extract contained peaks of heptapeptide (16 min) and pQFYRFamide (19 min) of roughly equal size (not shown). The pQFYRFamide peaks from both purifications were combined and run through a final TFA/ACN system. The major peak (12 to 12.5 min; Fig. 4A) was subjected to combined HPLC/mass spectrometry, revealing a molecular ion at 742 (Fig. 4B) in agreement with the calculated value of 742. Since this peptide eluted from the HPLC column at the same time as synthetic pQFYRFamide and had the same mass as pQFYRFamide, we conclude that it is pQFYRFamide.

Cardioactivity of pQFYRFamide

The effects of synthetic pQFYRFamide on the isolated heart of *H. aspersa* were compared with those of

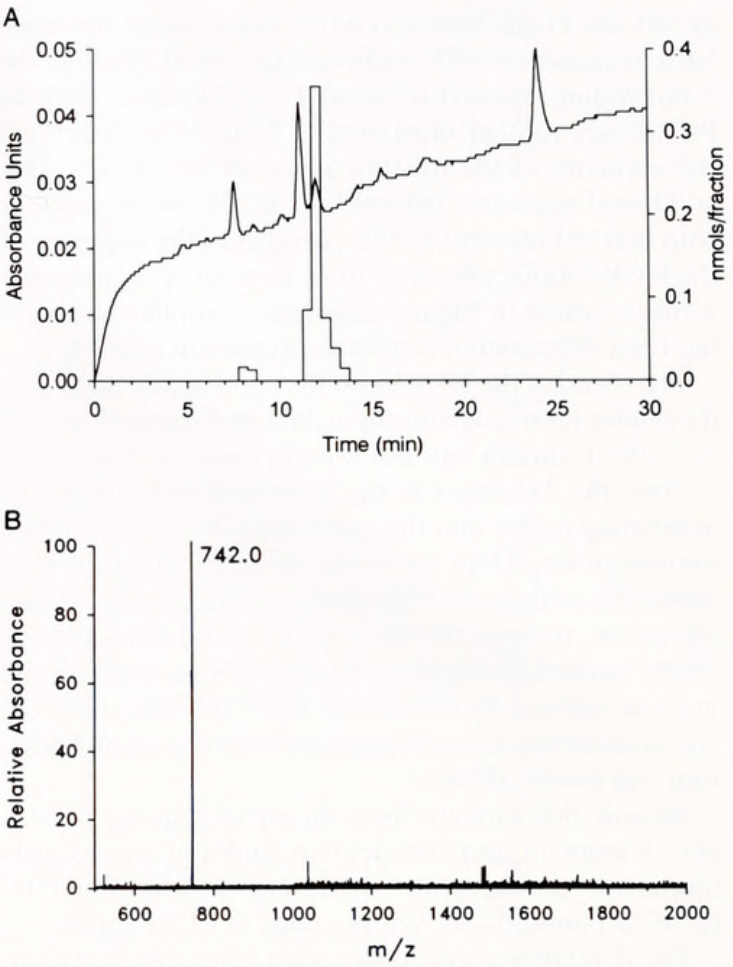


Figure 4. The purification and identification of pQFYRFamide. (A) The UV absorbance (214 nm) profile and a histogram of the immunoreactivity (determined with antiserum S253) in each fraction of the column effluent is shown for the final HPLC step (eluted with the TFA/ACN solvent system, see Methods). There is a delay of about 30 s between the UV recorder and the fraction collector for which no correction was made. (B) Mass spectral analysis of the main immunoreactive peak (12 min) from the fractionation illustrated in A. The observed m/z value (mass:charge ratio) of 742 corresponds to the calculated value for pQFYRFamide.

FMRFamide (Fig. 5). FYRFamide was about half as potent as FMRFamide, whereas pQFYRFamide was about 5 times more potent. Thus, blocking of the N-terminal by addition of pyroglutamic acid increases the potency of FYRFamide by 10-fold. Payza (1987) also noted that FMRFamide analogs with blocked N-terminals were more potent on the heart than those without.

Responses of identified neurons of Helix aspersa

Potassium current evoked in the C1 neuron. FMRFamide induces a slowly developing hyperpolarization of the C1 neuron that results from an increase in potassium conductance (Cottrell and Davies, 1987). In normal physiological solution (see Methods) pQFYRFamide evoked a response similar to that of FMRFamide (n = 5); *e.g.,* under voltage clamp at −45 mV, an outward cur-

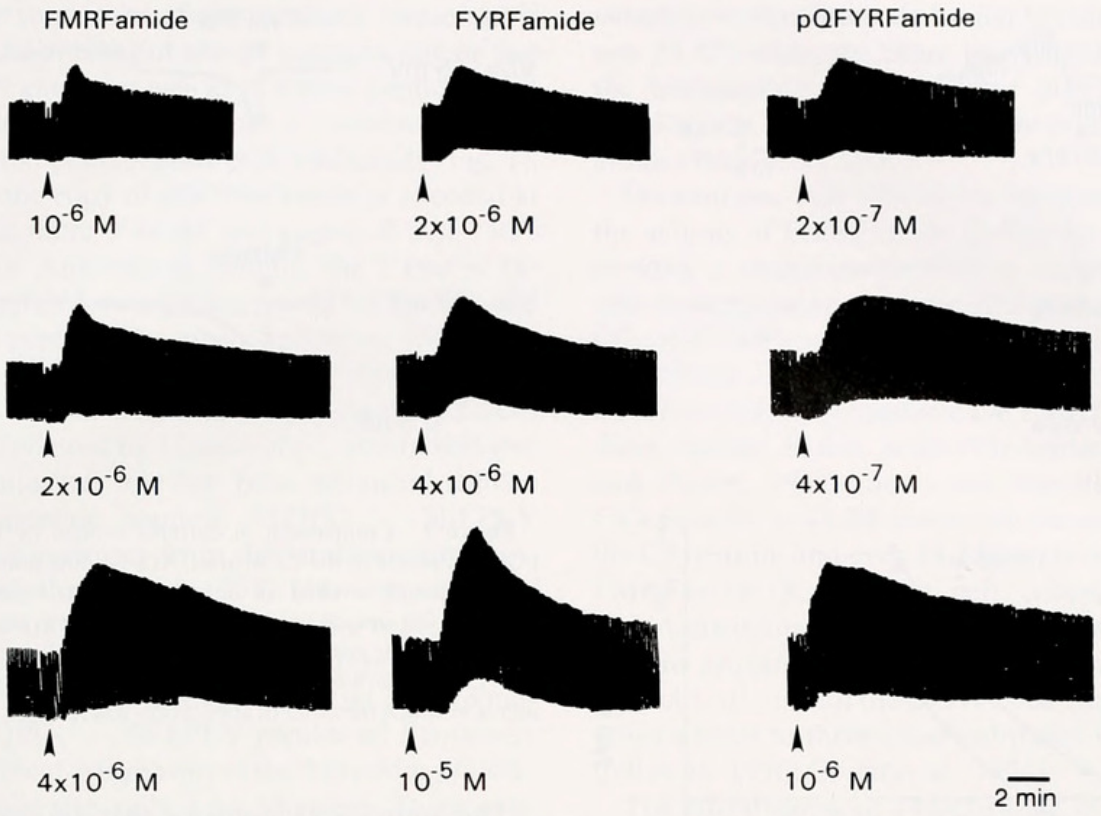


Figure 5. Representative recordings of the mechanical activity of an isolated perfused heart of *Helix aspersa*. A bolus (100 μl) of the indicated concentration of peptide was introduced into the perfusing stream at the arrow; this volume corresponds to about 30 s of flow.

rent accompanied by an increased membrane conductance was recorded (Fig. 6A). At a holding potential of −100 mV the direction of the current response was inward (bottom record, Fig. 6A). The relationships between evoked current and holding potential were compared in normal physiological solution (containing 5 m*M* K) and in a saline with reduced potassium (1.5 m*M*), as shown in Figure 6B. The shift in reversal potential produced by reducing the potassium concentration was about 30 mV, close to that predicted for a response mediated entirely by a change in potassium conductance. FMRFamide and pQFYRFamide both had EC_{50} values of about 2 μ*M*, but pQFYRFamide consistently produced a larger maximum effect (about 50% greater than that of FMRFamide).

Suppression of a voltage-dependent calcium current in the C1 neuron. FMRFamide reduces the voltage-dependent calcium current by up to 30% (Colombaioni *et al.*, 1985; Cottrell and Lesser, 1987). pQFYRFamide also reversibly reduced the amplitude of the Ca current and by a similar amount (n = 4; data not shown). The EC_{50} for pQFYRFamide was similar to that for FMRFamide, *i.e.*, about 5 μm.

Fast sodium current evoked in the C2 neuron. FMRFamide was shown to produce a rapidly developing inward current in the C2 neuron (Cottrell and Davies, 1987), an effect mediated by the direct activation of sodium channels (Cottrell *et al.*, 1990; Green *et al.*, 1994). A comparison of the effects of FMRFamide and pQFYRFamide, recorded under voltage clamp from the whole cell (see Fig. 7), clearly shows that pQFYRFamide fails to evoke this fast inward current (n = 7). Apparently, pQFYRFamide cannot activate the FMRFamide-gated sodium channel.

Potassium current evoked in the C2 neuron. Some C2 neurons, but not the example shown in Figure 7, responded to FMRFamide with a weak outward current in addition to the fast response. This outward current, like the slow hyperpolarization observed in many *H. aspersa* neurons, is due to an increased potassium conductance (see C1 neuron above). pQFYRFamide also, and consistently (n = 7), evoked a slow outward current (Figs. 7 and 8A) that was accompanied by an increase in membrane conductance (Fig. 8A). This response inverted close to the potassium equilibrium potential (Fig. 8B) in both normal and reduced potassium solutions (based on $[K_i]$ = 98 m*M*, Alvarez-Leefmans and Gamiño, 1982). It is, therefore, very similar to the slow increase in potassium conductance induced by FMRFamide and described above for the C1 neuron (Fig. 6; and Cottrell *et al.*, 1984); the EC_{50} values for pQFYRFamide and FMRFamide were similar—about 1 μ*M*.

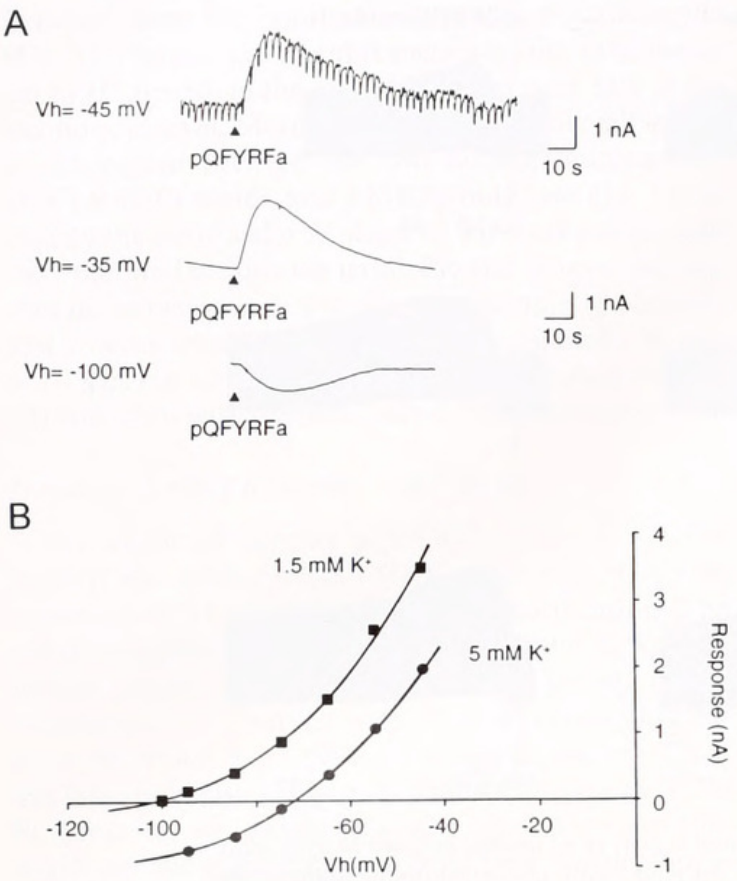


Figure 6. Effects of pQFYRFamide on a C1 neuron from the cerebral ganglia of *Helix aspersa*. (A) Examples of the currents evoked by the peptide at different holding potentials (Vh). In the top record, the voltage was periodically stepped to −50 mV to monitor the conductance; note that the current pulses in response to the −5 mV steps are about twice as large at the peak of the response as in the control. (B) Relationship between peak evoked current and holding potential for responses in physiological solutions containing normal (5 m*M*, circles) or reduced (1.5 m*M*, squares) concentrations of potassium. The data shown in the graph were taken from an experiment on a single C1 neuron. A second experiment gave the same result.

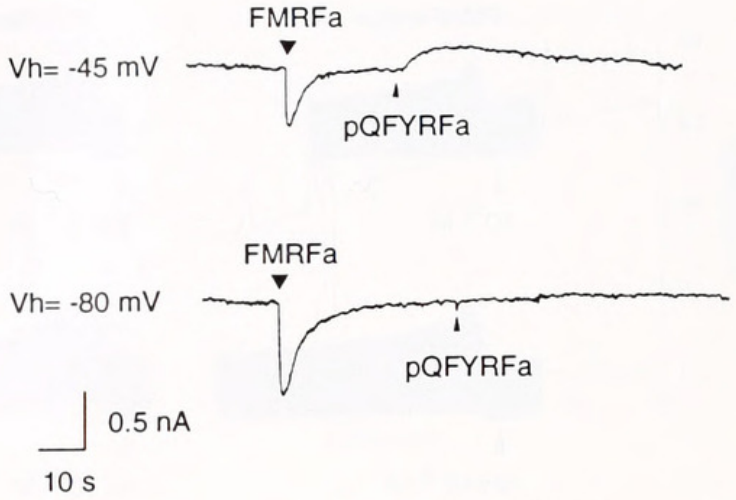


Figure 7. Comparison of currents evoked by FMRFamide and pQFYRFamide in the C2 neuron. At a holding potential of −45 mV, pQFYRFamide evoked an outward current, whereas FMRFamide evoked a fast inward current. Even at −80 mV, close to the reversal potential for the potassium response, no inward current in response to pQFYRFamide was seen. Identical results were obtained in experiments in which the order of application was reversed (not shown).

Discussion

We have sequenced the 5′ end of the cDNA encoding the FMRFamide precursor of *Helix aspersa*, and so the entire sequence is now complete. The general organization of this precursor is similar to that of the two other completely sequenced FMRFamide precursors: *Lymnaea stagnalis* (reviewed by Benjamin and Burke, 1994) and *Aplysia californica* (see Taussig and Scheller, 1986). The similarity is manifest in both the splicing pattern and in the linear arrangement of landmark sequences in the precursor.

The mRNA encoding the FMRFamide precursor in all three species is composed of at least two exons, and in all three the splice junction is in a roughly similar position. In both *Lymnaea* and *Helix* the first exon of the FMRFamide precursor is alternatively spliced to give another neuropeptide precursor (that of the FLRFamide-related heptapeptides), but no alternatively spliced product has ever been found in *Aplysia*.

The amino acid segments that are processed to become the individual mature peptides are not distributed at random in the precursors, rather, they are clustered according to their sequences in two domains. First, the C-terminal end of the precursor (always downstream of the tetrabasic sequence, RKRR) is the site of repeated segments, each comprising a FMRF sequence with its processing signals and acidic spacers. Second, the N-ter-

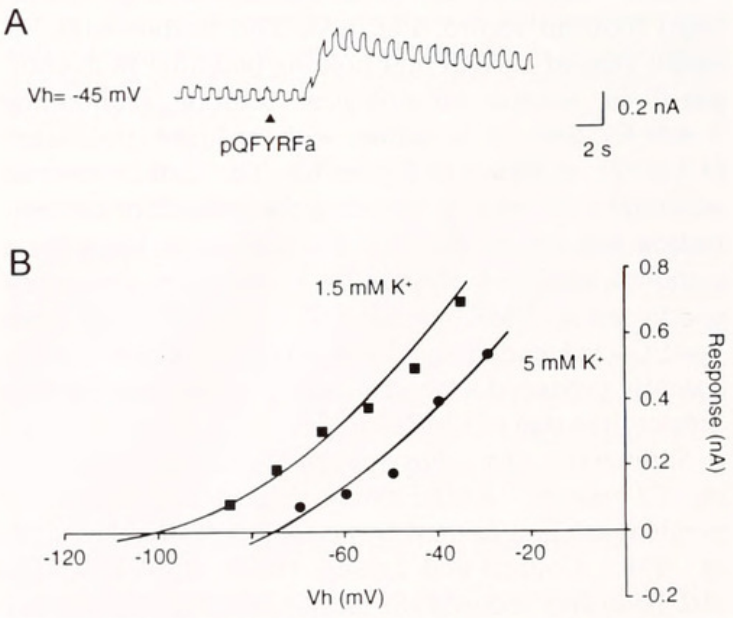


Figure 8. (A) The effect of pQFYRFamide on the C2 neuron at −45 mV is accompanied by an increase in conductance as indicated by the increase in current pulses evoked by periodically stepping the voltage to −80 mV. (B) Relationship between peak current and holding potential (Vh) for responses evoked in physiological solutions containing either the normal (5 m*M*, circles) or reduced (1.5 m*M*, squares) level of potassium in an experiment on a single C2 neuron.

minal domain (upstream of the tetrabasic sequence) is devoid of FMRFamide but always contains one or two copies of FLRFamide, as well as of related peptides. The related peptide in *H. aspersa* and *C. nemoralis* is the newly discovered pentapeptide pQFYRFamide (Fig. 1). In *Lymnaea,* one copy of pQFYRFamide is encoded at the level of the more 3′ of the two copies of pQFYRFamide in *Helix.* And even in *Aplysia,* the 5′ end of the FMRFamide mRNA encodes a copy of FLRFamide and a long, related peptide (Greenberg and Price, 1992).

The functional significance of the tetrabasic site and the sequences surrounding it has been considered from time to time (reviewed by Linacre *et al.,* 1990). In *Lymnaea,* a physiological role has been advanced for the acidic dodecapeptide segment SEQPD . . . SEEPLY that occurs just upstream from the tetrabasic site (Linacre *et al.,* 1990; Burke *et al.,* 1993). Our comparison of the *Polydontes* and *H. aspersa* precursors bears on this notion of function. Of the 23 amino acids preceding the tetrabasic site in these two land snails (and corresponding to the SEQPD . . . SEEPLY peptide of *Lymnaea*), only 5 are identical. Moreover, of the 23 residues *following* the tetrabasic site, only 3 are identical. These nonconserved sections of the precursor are, therefore, not likely to have a specific function common to all pulmonates, let alone all molluscs.

In contrast, the sequence of amino acids in the tetrabasic site seems to be conserved among pulmonates and opisthobranchs; moreover, it fits the consensus motif (Arg-Xaa-Lys/Arg-Arg) of a site of action for a furin-like endopeptidase (Hosaka *et al.,* 1991). The involvement of furin-like cleavage in neuropeptide processing was first demonstrated for the egg-laying hormone of *A. californica* (Newcomb *et al.,* 1988; Fisher *et al.,* 1988). Putative furin sites have since been observed in the myomodulin precursors of *Aplysia* (Miller *et al.,* 1993) and *L. stagnalis* (Kellett *et al.,* 1996); and the first cleavage seems to occur at such a site during the processing of the heptapeptide precursor in *H. aspersa* (Cottrell *et al.,* 1994). As for the tetrapeptide precursor, Linacre *et al.* (1990) had already suggested that the FLRFamide and FMRFamide domains could be separated by cleavage at the RKRR sequence. This conjecture was later elaborated by Greenberg and Price (1992), and it appears to be a reasonable working hypothesis.

If, however, this hypothesis is correct, and molluscan FMRFamide precursors are first processed into N- and C-terminal domains, then the products of the two domains might be functionally, and not merely structurally, distinct. In search of such a distinction, we compared the actions of pQFYRFamide (restricted to the N-terminal domain) with those of FMRFamide (restricted to the C-terminal domain) on the heart and selected neurons of *H. aspersa.*

The activity of pQFYRFamide on the isolated *Helix* ventricle was qualitatively similar to that of FMRFamide and FLRFamide, the other two peptides derived from the tetrapeptide precursor. But pQFYRFamide, like FLRFamide, is about 5-fold more potent than FMRFamide (Price *et al.,* 1990).

On neurons, pQFYRFamide has some, but not all of the actions of FMRFamide. It mimics FMRFamide in evoking a potassium-dependent outward current and also in suppressing a voltage-activated calcium current. But pQFYRFamide—unlike FMRFamide—does not activate the fast inward sodium current in neuron C2, and thus it must not activate the FMRFamide-gated sodium channel. In fact, neither the heptapeptides (Cottrell and Davies, 1987), nor acetyl-Phe-Nle-Arg-Phe-NH_2, FYRFamide, or FLRF evoke this fast sodium current in the C2 neuron; and even FLRFamide is less potent than FMRFamide (K.A. Green, pers. comm.). Recently, the cDNA encoding this FMRFamide-gated sodium channel was sequenced and expressed in frog oocytes (Lingueglia *et al.,* 1995); these expressed channels had properties similar to those observed in the C2 neuron (Cottrell *et al.,* 1990; Green *et al.,* 1994).

The effectiveness of FMRFamide in evoking the inward sodium current—in contrast to the ineffectiveness of pQFYRFamide and FLRFamide—indicates that the recognition site for the fast ligand-gated response has a high level of specificity. More important, it shows that the pharmacological activity of the major peptide at the N-terminal portion of the precursor protein is different from the activity of the major peptides at the C-terminal portion, at least at one site.

In summary, we have sequenced the 5′ end of the cDNA for the FMRFamide precursor of *H. aspersa.* When the FMRFamide precursors from different species were compared, the overall level of similarity varied with the phyletic distance between the species. But in every case, the sequences incorporating the FMRFamide-related peptides and the tetrabasic recognition site, as well as the organization of the precursor, were strongly conserved. Noting the putative peptide pQFYRFamide encoded on this cDNA segment, we searched for and subsequently identified this peptide in extracts of ganglia. Mass spectroscopy confirmed the predicted sequence and post-translational modifications (*i.e.,* amidation and cyclization of glutamine to pyroglutamic acid). pQFYRFamide had effects similar to those of FMRFamide on the heart and to an extent on neurons. But this novel pentapeptide did not activate the neuronal FMRFamide-gated sodium channel.

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