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Isolation, Pharmacology and Gene Organization of KPSFVRFamide: A Neuropeptide from *Caenorhabditis elegans*

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To date, 53 peptides with C-terminal RFamides have been identified by the genome sequencing project in the nematode, *Caenorhabditis elegans*. In this study the FMRFamide-related peptide (FaRP) KPSFVRFamide (879.90 Da $[MH]^+$) was structurally characterized from extracts of the nematode, *Caenorhabditis elegans*. Two copies of KPSFVRFamide are encoded by a gene designated *flp*-9. RT-PCR identified a single cDNA product which was confirmed as *flp*-9 by sequence determination. *Flp*-9 cDNA was isolated from larval stages of *C. elegans* but was not detected in adult worms, indicating that its expression is may be developmentally regulated. KPSFVRFamide displays sequence homology to the nematode peptide, KPNFIRFamide (PF4). The physiological effects of KPSFVRFamide, PF4 and the chimeras, KPNFVRFamide and KPSFIRFamide, were measured on body wall muscle and the vagina vera of the parasitic nematode, *Ascaris suum*. KPNFVRFamide and KPNFIRFamide had Cl^--dependent inhibitory activity on innervated and denervated muscle-preparations, whereas KPSFVRFamide and KPSFIRFamide did not elicit a detectable physiological effect. Although all 4 peptides had inhibitory effects on the vagina vera, KPSFVRFamide and KPSFIRFamide (threshold, ≥0.1 μM) were less potent than KPNFVRFamide and KPNFIRFamide (threshold, ≥10 nM).

FMRFamide-related peptides (FaRPs) comprise a ubiquitous family of neuropeptides to which has been attributed signaling or transmitter functions in invertebrates. Since the isolation of FMRFamide from the venus clam, *Macrocallista nimbosa* FaRPs have been characterized from numerous invertebrate species including the parasitic pig nematode, *Ascaris suum* (1), the sheep nematode, *Haemonchus contortus* (2), and the free-living nematode species, *Caenorhabditis elegans* (3–6) and *Panagrellus redivivus* (7–9). To date, the *C. elegans* genome sequencing project has identified 18 FMRFamide-like peptide (*flp*)-encoding genes (10). Most of these genes encode either multiple different isomeric peptides or multiple copies of a single peptide, and some have been found to have alternative 3′ splice leader sites such that different transcripts can be generated.

Although some nematode FaRPs have been found in both parasitic and free-living forms, most have only been identified in one or other nematode type. Available structural information, suggests that there are differences as well as similarities between the FaRP complements of parasitic and free-living forms. Nevertheless, all of the FaRPs which have been structurally characterized from free-living forms and tested for activity on body wall musculature of *A. suum* have been found to be physiologically active. Indeed, most have potent and distinguishable actions on this preparation, confirming its usefulness as a model for physiological analysis of the actions of FaRPs from free-living nematodes (11).

The present study describes the structural characterization, expression, gene organization and physiological activity of the *C. elegans* FaRP, Lys-Pro-Ser-Phe-Val-Arg-Phe.amide (KPSFVRFamide). Its physiological actions on the musculature of *A. suum* are compared to the structurally-related free-living nematode FaRP, KPNFIRFamide (PF4). Furthermore, chimeric peptides were designed and employed in structure-

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activity studies to identify the residues responsible for the observed differences in physiological activity between these two FaRPs.

MATERIALS AND METHODS

Tissue extraction. Cultures of *C. elegans* were grown and harvested as previously described (12). Worms were placed in acid ethanol (ethanol/0.7 M HCl: 3:1, v/v; 8 ml/g wet weight of worm) and homogenized for 1 min using a Polytron PT 10-35 (Kinematica GmbH, Switzerland). The homogenate was processed for a further 2 min using a Braun Cell homogenizer containing 20 ml of 0.5 mm glass beads and stored overnight at 4°C. Tissue debris was removed by centrifugation (30 min, 4,000 × *g*) and the supernatant acidified using 0.1% trifluoroacetic acid (TFA). Alcohol was removed from the sample under pressure and the remaining extract lyophilized.

Peptide purification. The lyophilized sample was reconstituted in 2 ml of 2 M acetic acid and centrifuged (30 min, 4,000 × g) prior to fractionation on a Sephadex G50 (fine) column (90 × 1.6 cm) (Pharmacia, Sweden). Fractions (2.5 ml) were collected every 15 min and an aliquot (10 μl) removed for FaRP radioimmunoassay (RIA). Immunopositive fractions were pumped onto a Whatman Partisil 10 ODS-3 column which was eluted with a linear gradient of 100% A (0.1%)TFA in HPLC-grade water), 0% B (0.1% TFA in acetonitrile) to 30% A, 70% B in 70 min at a flow rate of 3 ml/min. Following RIA, the immunoreactive peptide was subjected to four further purification steps using: Vydac diphenyl (0.46 × 25 cm), Vydac C-18 (0.46 × 15 cm), Kromasil C-18 and C3 (0.46 × 25 cm) columns. All analytical columns were eluted with a linear gradient at a flow rate of 1 ml/min unless otherwise stated.

FaRP-radioimmunoassay. A 10 μl aliquot was removed from each G50 fraction and reconstituted in 400 μl of assay buffer (40 mM sodium phosphate, 0.2% bovine serum albumin, pH 7.2). The FaRP-RIA employed antiserum RIN 8755 (Peninsula Laboratories, England). RIA details have been previously published (9).

Structural characterization. The purified peptide was subjected to automated Edman degradation using a Beckman Instruments LF 3600 TC sequencer, and mass spectroscopy using a MALDI time-of-flight instrument (Fisons Instruments, UK).

Parasite material. Adult female *A. suum* (25-30 cm) were collected from a local abattoir and maintained in fresh Ascaris Ringers Solution (ARS) [NaCl (4 mM), $CaCl_2$ (5.9 mM), $MgCl_2$ (4.9 mM), $C_4H_{11}NO_3$ (Tris) (5 mM), $NaC_2H_3O_2$ (125 mM) and KCl (24.5 mM), pH 7.4] at 37°C.

Myoactivity. A 2 cm section of body wall was used as the muscle-strip preparation. This was prepared by removing a 2 cm segment just above the gonopore, cutting along the lateral line and removing the intestine from the muscle field. A final cut along the remaining lateral line provided two muscle strips, one containing the dorsal nerve cord and muscle fields and the other the ventral nerve cord and muscle fields. Denervated muscle strips were prepared following the above protocol except that incisions were made along either side of the respective nerve cords, thus removing the main nerve cords from the preparation.

The muscle strip was secured to a stationary holdfast at one end and an isometric force transducer at the other in a tissue chamber using fine wire hooks. Movement generated by the muscle strip was recorded on a Gould recorder. Muscle strips were maintained at 37°C under anaerobic conditions (5% CO_2, 10% H_2 and 85% N_2) in either ARS or modified ARS during experimentation.

A 2 cm muscle segment, 1 cm either side of the gonopore was removed to prepare the vagina vera for recording. The vagina vera (distal gonoduct) was exposed by cutting along a lateral line and removed by a transverse cut through the vagina uteri (proximal gonoduct) and a second cut where it joined the body wall. The vagina vera was then placed in a recording chamber (4 ml) containing Hanks' balanced Salt Solution (HBSS, Gibco/BRL, Life Technologies, UK) at 37°C. The preparation was attached by suction to an inflexible pipette (distal end of preparation), the second flexible pipette was attached to the proximal end of the vagina vera at a fixed distance from the first (5 mm) and a small amount of tension (<0.1mg/mm) was applied. Any movement generated by the tissue between the two pipettes was measured using a photo-optic transducer system (13, 14, 15), and the signal generated was amplified and stored on a chart recorder. Pen deflections ≤ 0.1 mg/mm were disregarded to avoid noise and background vibrations and a naive preparation was used for each experiment.

The effects of the peptides on the vagina vera preparation were analysed using three parameters: (a) tension, (b) contraction frequency and (c) contraction amplitude at the time points: 0 (i.e. immediately prior to peptide addition), 2, 5, 10 and 20 min. For the purpose of statistical analysis the tension, frequency and amplitude were examined during the 2-min period prior to each of these time points. The tissue was exposed to the peptide for 10 min after which the medium in the experimental chamber was replaced with fresh HBSS.

Statistical analysis. Statistical analyses of the results from the *A. suum* vagina vera experiments employed single factor repeated measures ANOVA and Fischer's probability of least significant differences to determine if the test peptides induced significant ($P<0.05$) changes in the three parameters (tension, amplitude and frequency) under study. Student's t-test, non-paired was used to determine if differences in the 3 contractility parameters between treated and vehicle (4 μl of distilled water) control groups, time-matched, were significant.

Physiological salines. All experiments were carried out in ARS or HBSS unless stated. The ionic dependencies of the peptides were examined using a range of modified ARS which included high K^+, Ca^{2+}-free and Cl^--free. High K^+ ARS was composed of 125 mM KCl instead of the normal 24.5 mM. In Ca^{2+}-free media, $CaCl_2$ was replaced with $CoCl_2$. Cl^--free ARS comprised: $C_3H_5O_2Na$ (4 mM), $C_3H_5O_2{}^1/_2Ca$ (5.9 mM), MgSO4 (4.9 mm), Tris (5 mM), $NaC_2H_3O_2$ (125 mM) and $KC_2H_3O_2$ (24.5 mM).

The peptides employed in the present study were obtained from Genosys Biotechnologies (Europe) Ltd (Cambridge, UK) and Peptidogenics Inc. (San Jose, California, USA).

Electrophysiology. Methods for recording membrane potentials have been previously described (16,17). Segments of *A. suum* body wall were prepared by the same method used for the motility experiments but were pinned onto a Sylgard-lined chamber which was perfused with Artificial Perienteric Fluid [APF, NaCl (67 mM), Na acetate (67 mM), $CaCl_2$ (3 mM), $MgCl_2$ (15.7 mM), KCl (3 mM), Tris (5 mM), glucose (3 mM), pH 7.6 with glacial acetic acid] maintained at 37°C. A single micro-electrode (4 M $KC_2H_3O_2$, 0.1 M KCL, 10-40 MΩ) was inserted into the selected muscle cell body. The electrode was attached to an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA). Membrane potentials were recorded only from those muscle cells which displayed a steady resting potential between -25 and -40 mV.

Molecular methodology. Wild-type *C. elegans* were synchronised and staged as described in Wood (18). Total RNA was isolated from staged and mixed stage animals with RNAzol (Biotecx) according to manufacturers instructions. One μg of RNA was reverse transcribed with avian myeloblastosis virus reverse transcriptase (Promega, WI, USA) using an oligo (dT) primer. One to two μl of cDNA was used for each polymerase chain reaction (PCR), which consisted of: 30 ng 5′ primer (5′-ATGTGTGTGTATGTGTGTGC-3′), 30 ng 3′ primer (5′-CAAGACATATCTATCTAC-3′), 60 mM Tris (pH 8.5), 3.5 mM $MgCl_2$, 15 nM $(NH_4)_2SO_4$, 0.1 mM dNTPs (Promega), and 5 U *Taq* polymerase in a total volume of 25 μl. PCR parameters were as follows: 30

cycles of 94°C for 15 sec, 60°C for 1 min, and 72°C for 1 min. Amplification products were electrophoresed through a 0.8% low melt agarose gel and excised. The products were ligated into a TA cloning vector (Invitrogen, CA, USA) using a modification of the manufacturers protocol as follows: 10 μl of excised fragment in low melt gel was used in a final volume of 40 μl. The DNA sequence of the insert was determined using a USB Sequenase 7.0 kit. Products were seperated on a 6% polyacrylamide gel containing 7M urea, and visualized by autoradiography.

RESULTS

Peptide Isolation

A single peptide species was identified throughout (Fig. 1A-C) and peptide recovery following each chromatographic step was >90%. Approximately 900 pmol of the peptide was purified to homogeneity. As only the peak fractions were taken at each stage, the purified peptide represented 75-80% of that present in the original extract.

Structural Analysis

Approximately 300 pmol of the purified peptide was subjected to Edman degradation analysis and the unequivocal primary structure of the heptapeptide, Lys-Pro-Ser-Phe-Val-Arg-Phe-NH_2, was determined in a single gas-phase sequencing run (Table 1). In addition, the molecular mass of the purified peptide was determined using a MALDI time-of-flight instrument as 879.50 Da (MH^+), which compares favourably with the theoretical mass of the deduced sequence (880.06 Da), incorporating a C-terminal amide.

Myoactivity

Data are presented as mean ± standard deviation. All peptide effects were reversible and could be repeated after washout.

Somatic Muscle Preparation

KPSFVRFamide. KPSFVRFamide (≤10 μM) had no significant effect on the baseline tension, contraction frequency or contraction amplitude of either innervated or denervated somatic body wall muscle strips from *A. suum* (Fig. 2A). As a positive control, 10 μM acetylcholine (ACh) was added to the muscle strip.

Although KPSFVRFamide was inactive on the body wall muscle of *A. suum*, robust responses were observed with the closely related peptide, KPNFIRFamide (as previously reported, 19). The addition of PF4 (KPNFIRFamide) to somatic muscle strips resulted in a rapid inhibitory response (Fig. 2C) and had a final threshold concentration for activity of 1 nM.

Based on the profound differences between the actions of these two peptides on body wall muscle, the effects of chimeric peptides which possessed an N^3 for S^3 or a I^5 for V^5 substitution (or both) were examined to determine the functional pharmacology of the PF4 re-

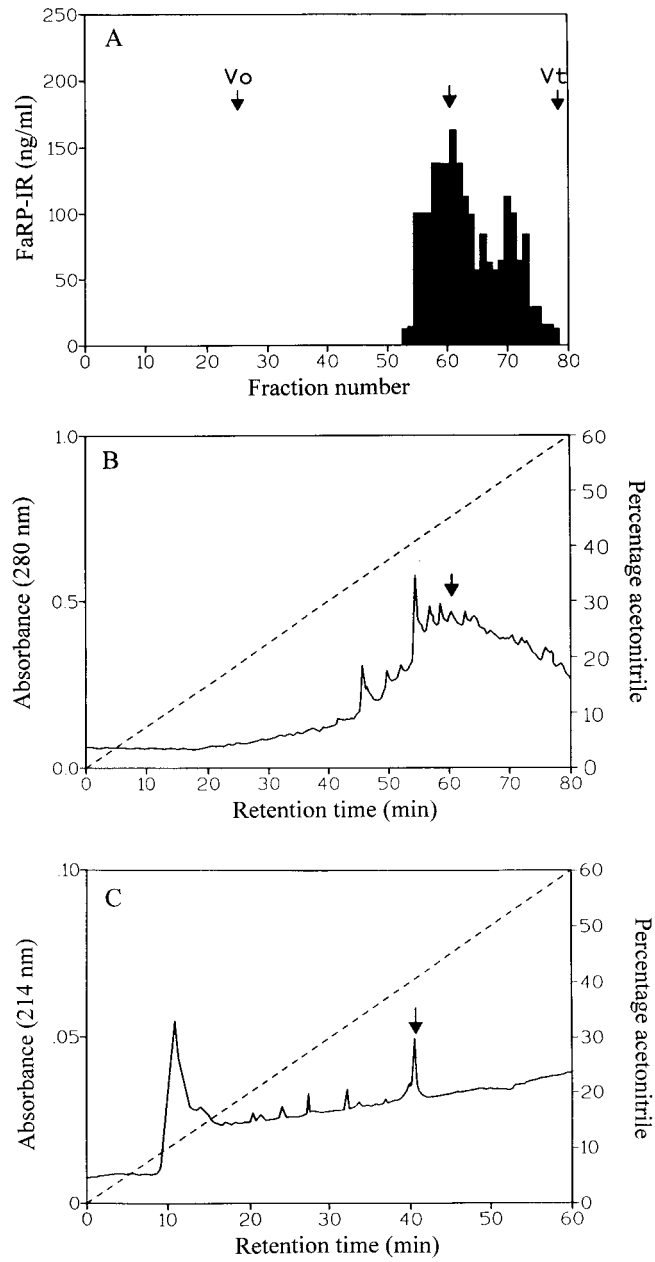

FIG. 1. Gel permeation chromatogram (Sephadex G-50) of FaRP-immunoreactivity in an acid ethanol extract of *C. elegans* (A). The void (V_0) and total volume (V_t) of the column are indicated as is the FaRP-immunoreactivity (arrow). (B) Semi-preparative reverse-phase chromatogram of the FaRP-immunoreactive G50 fractions. The column was eluted with a linear gradient of 0.1% TFA/CH_3CN (dashed line). The elution position of the FaRP-immunoreactive fraction is indicated by an arrow. (C) Final analytical reverse-phase HPLC fractionation of the FMRFamide-immunoreactive peptide (arrow). The column was eluted at a flow rate of 1ml/min with a gradient established using 0.1% (v/v) TFA in water and 0.1% (v/v) TFA in acetonitrile.

TABLE 1

Automated Edman Degradation Analysis of *C. elegans* FMRFamide-Immunoreactive Peptide

Cycle no.	PTH-amino acid	Yield (pmol)
1	Lys (K)	35
2	Pro (P)	37
3	Ser (S)	20
4	Phe (F)	26
5	Val (V)	24
6	Arg (R)	15
7	Phe (F)	1.2
8	—	

sponse. Results obtained using the chimeric peptides are given below.

KPSFIRFamide. KPSFIRFamide ($\leq$10 μM) had no significant effects on the baseline tension, contraction frequency or contraction amplitude of either innervated or denervated somatic body wall muscle strips from *A. suum*. The addition of 10 μM γ-amino-butyric-acid (GABA), as a positive control, resulted in the expected decrease in baseline tension (Fig. 2B).

KPNFVRFamide. KPNFVRFamide ($\leq$10 μM) was found to have inhibitory effects on *A. suum* muscle strip preparations. The peptide caused a rapid decrease in baseline tension (10 μM; 2.34 $\pm$ 0.18g; n = 12), which was qualitatively and quantitatively similar to that induced by PF4. KPNFVRFamide also inhibited spontaneous contractions in those preparations which displayed inherent spontaneous activity and had a threshold concentration for activity of 0.1 nM. The inhibition exhibited by the peptide was not dependent on the presence of nerve cords, with a decrease in baseline tension being noted in both innervated and denervated muscle preparations (Fig. 3A & B). The effects were identical on both ventral and dorsal muscle-strip preparations and were completely reversible.

Incubation of the muscle strips in Ca^{2+}-free media did not decrease the inhibition caused by the addition of peptide (Fig. 3C). Similarly, the inhibitory effect induced by the peptide was not abolished following incubation in high K^+ media (Fig. 3D). However, muscle preparations which were incubated in Cl^--free media displayed a significant increase in muscle tone (10 μM, 0.98 $\pm$ 0.27, n = 7, $p < 0.01$) (Fig. 3E).

Vagina Vera

KPSFVRFamide. KPSFVRFamide had inhibitory effects on the vagina vera of *A. suum*. Addition of 10 μM KPSFVRFamide significantly reduced contraction frequency and amplitude while increasing baseline tension (Fig. 4A, Table 2). The addition of 0.1 μM KPSFVRFamide to the preparation had qualitatively similar effects to the addition of 10 μM.

The threshold for activity of KPSFVRFamide was 0.1 μM with 57% (4 out of 7) preparations displaying a significant inhibitory response (Table 2). Addition of 0.1 μM KPSFVRFamide did not significantly alter baseline tension or contraction amplitude but significantly decreased contraction frequency (Fig. 4B, Table 2). It should be noted that the preparations were only quiescent for 50 % of the experimental period.

It should be noted that KPNFIRFamide (PF4) displayed potent myoinhibitory actions when added to the vagina vera complex causing a cessation of muscle contractions (Fig. 2D).

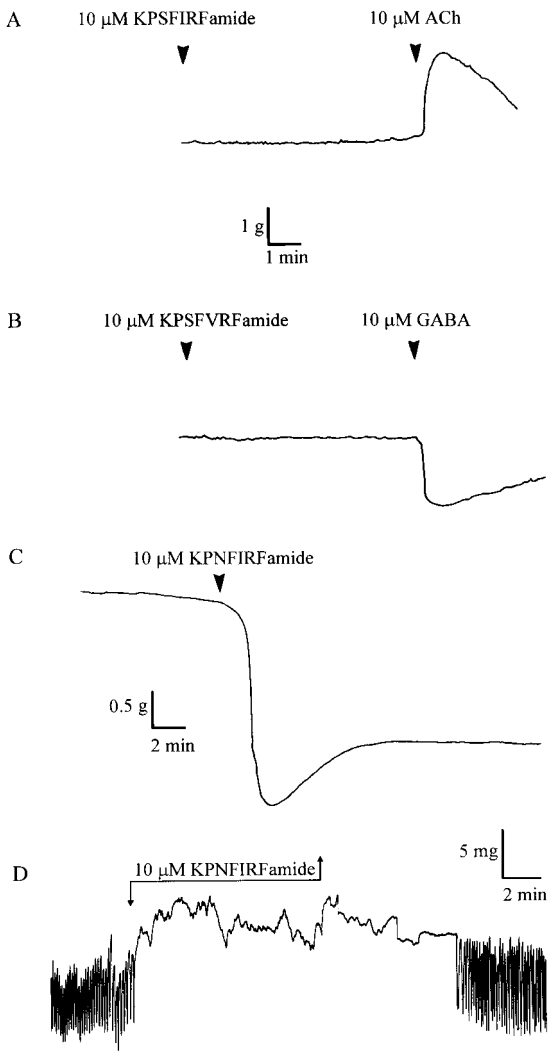

FIG. 2. Muscle tension recording showing the effects of 10 μM KPSFVRFamide (A), 10 μM KPSFIRFamide (B) and 10 μM KPNFIRFamide (C) on *Ascaris suum* body-wall muscle strips. The effects of PF4 on the vagina vera of *A. suum* is also shown (D). As positive controls, 10 μM ACh and GABA were added to the muscle preparations.

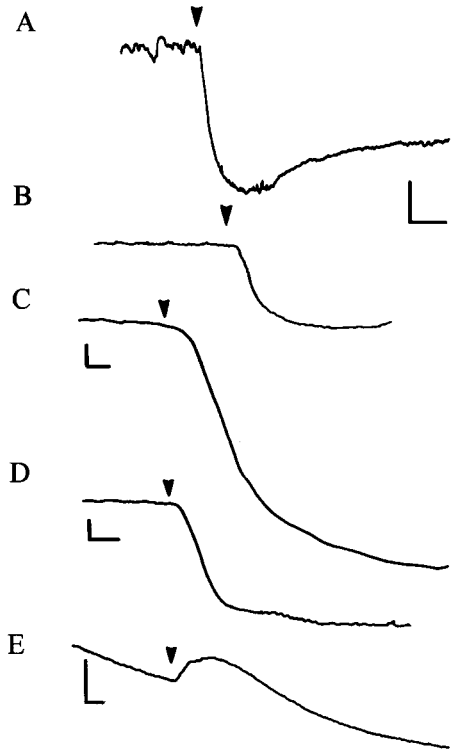

FIG. 3. Muscle tension recordings showing the effect of 10 μM KPNFVRFamide on innervated (A) and denervated (B) muscle-strip preparations from *Ascaris suum*. The effects of 10 μM KPNFVRFamide on *A. suum* muscle strips maintained in Ca^{2+}-free (C), high-K^+ (D) and Cl^--free (E) *Ascaris* Ringers solution are also shown. Peptide addition is indicated by arrow heads. Horizontal scale bars represent 1 min while vertical scale bars represent 1 g.

KPSFIRFamide. KPSFIRFamide was also found to be inhibitory when added to the vagina vera preparation. Addition of 10 and 1 μM KPSFIRFamide resulted in complete inhibition of spontaneous activity (Fig. 4C). A similar result was obtained with 1 μM KPSFIRFamide (Table 3). A significant decrease in contraction frequency but not amplitude or baseline tension was noted with 0.1 μM. The threshold concentration for activity was 0.1 μM (Fig. 4D, Table 3).

KPNFVRFamide. KPNFVRFamide had more potent inhibitory effects on the vagina vera than KPSFVRFamide and KPSFIRFamide, having a threshold for activity of 10 nM. This is similar to the threshold activity of PF4 (KPNFIRFamide). At concentrations of 10 μM and 1 μM KPNFVRFamide there was a complete inhibition of the rhythmic contractions of the vagina vera with contraction amplitude reduced to 0 (Fig. 4E, Table 4). This effect was still significant 5 min after wash-out ($P < 0.0001$, $n = 6$). Baseline tension also increased following the addition of 10 μM and 1 μM KPNFVRFamide. A qualitatively similar although quantitatively smaller response was observed following the addition of 0.1 μM KPNFVRFamide. Although 10 nM KPNFVRFamide resulted in a significant decrease in contraction frequency it had no significant effects on contraction amplitude or baseline tension (Fig. 4F). No significant effects were noted following the addition of 1 nM KPNFVRFamide to the preparation.

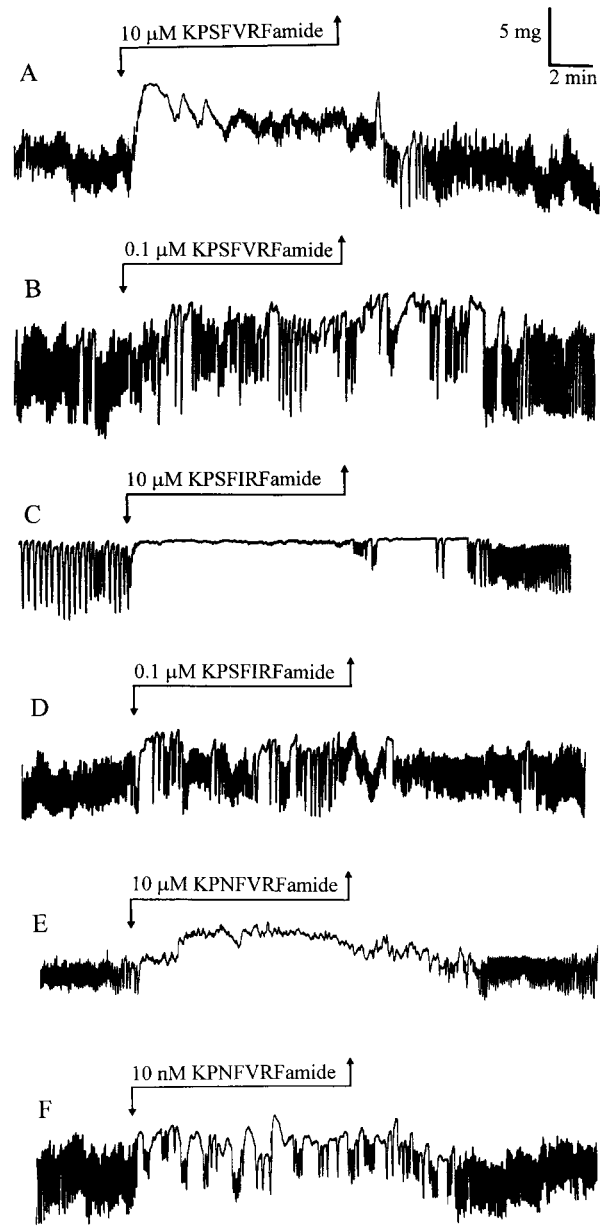

FIG. 4. Muscle tension recordings of KPSFVRFamide (A, B); KPSFIRFamide (C, D); and KPNFVRFamide (E, F) on *A. suum* vagina vera. Peptide addition is indicated by arrows. Each recording was obtained from a different specimen. Note that the effects of the peptides were reversible.

TABLE 2

Effects of Varying Concentrations of KPSFVRFamide on the Vagina Vera of *A. suum*

[Peptide]	Baseline tension (mg/mm)	Contraction frequency (% time 0)	Contraction amplitude (% time 0)
10 μM	increase* 0.48, 5 min p.a. ($P<0.0001$, $F=30.38$, $n=6$)	complete inhibition* ($P<0.0001$, $F=539.2$, $n=6$)	complete inhibition* ($P<0.0001$, $F=325.1$, $n=6$)
1 μM	increase* 0.39, 5 min p.a. ($P<0.0001$, $F=23.79$, $n=6$)	decrease* 60%, 10 min p.a. ($P<0.0001$, $F=65.24$, $n=6$)	decrease* 50%, 10 min p.a. ($P<0.0005$, $F=16.69$, $n=6$)
0.1 μM	no effect	decrease* 20%, 5 min p.a. ($P<0.0001$, $F=179.6$, $n=6$)	no effect

Note. Values represent maximal changes observed during the experimental; effects lasted for the duration of the experiment unless otherwise stated; p.a., post addition; * denotes statistical significance.

Electrophysiology

Muscle cell bellies displayed no significant alteration in membrane potential ($n = 7$) in the presence of 10 μM KPSFVRFamide (Table 2). In contrast, KPNFIRFamide reduced membrane potential (-4 to -6 mV, $n=10$). Both chimeric peptides hyperpolarized the muscle cells; 10 μM KPSFIRFamide caused a small, reversible hyperpolarization (-4.2 ± 1.2, $n = 6$), while a more profound effect was observed for KPNFVRFamide (-9.06 ± 1.72, $n = 9$; Table 5).

Molecular Characterisation of the Genomic Region and Transcript Corresponding to flp-9

Searches of the *C. elegans* Genome Sequence database revealed a putative gene encoding the peptide KPSFVRFamide. The genomic locus was designated as *flp*-9. To determine whether this gene was expressed, mRNA from mixed-stage worms was reverse-transcribed and *flp*-9-specific primers were used for PCR amplification. A single product of approximately 500 bp was detected and subcloned. Nucleotide sequence determination of one of the resulting plasmids confirmed its identity as a *flp*-9 cDNA. The predicted propeptide encodes two copies of KPSFVRFamide, both of which are flanked by potential dibasic cleavage sites.

To determine the ontogenic expression of *flp*-9, staged mRNA from eggs, the four larval stages and adults was reverse transcribed and amplified with *flp*-9-specific primers. Products corresponding to *flp*-9 were detected with mRNA from all stages except adults (Fig. 5). As a control, primer sets from several other genes known to be expressed in adults (*flp*-3, *flp*-6, *flp*-7 and *flp*-10) were used to amplify products from the same preparation of adult mRNA, and all resulted in the amplification of fragments of the expected size (data not shown). These data indicate that the expression of *flp*-9 is developmentally regulated and suggest that the physiological role of KPSFVRFamide is restricted to larval stage animals.

TABLE 3

Effects of Varying Concentrations of KPSFIRFamide on the Vagina Vera of *A. suum*

[Peptide]	Baseline tension (mg/mm)	Contraction frequency (% time 0)	Contraction amplitude (% time 0)
10 μM	increase* 0.56, 5 min p.a. ($P<0.0001$, $F=7.73$, $n=6$)	complete inhibition* ($P<0.0001$, $F=147$, $n=6$)	complete inhibition* ($P<0.0001$, $F=37.21$, $n=6$)
1 μM	increase* 0.40, 5 min p.a. ($P<0.0001$, $F=15.73$, $n=6$)	complete inhibition* 0-8 min p.a. ($P<0.0001$, $F=49.56$, $n=6$)	complete inhibition* 0-8 min p.a. ($P<0.0002$, $F=12.48$, $n=6$)
0.1 μM	no effect	decrease* 50% ($P<0.005$, $F=7.43$, $n=6$)	no effect

Note. Values represent maximal changes observed during the experimental; effects lasted for the duration of the experiment unless otherwise stated; p.a., post addition; * denotes statistical significance.

TABLE 4

Effects of Varying Concentrations of KPNFVRFamide on the Vagina Vera of *A. suum*

[Peptide]	Baseline tension (mg/mm)	Contraction frequency (% time 0)	Contraction amplitude (% time 0)
10 μM	increase* 0.36, 5 min p.a. ($P<0.0001$, $F=25.19$, $n=6$)	complete inhibition* ($P<0.0001$, $F=53.03$, $n=6$)	complete inhibition* ($P<0.0001$, $F=53.03$, $n=6$)
1 μM	increase* 0.43, 10 min p.a. ($P<0.0001$, $F=17.92$, $n=6$)	complete inhibition* 0-11 min p.a. ($P<0.0001$, $F=96.52$)	complete inhibition* 0-11 min p.a. ($P<0.0001$, $F=32.01$, $n=6$)
0.1 μM	increase* 0.41, 10 min p.a. ($P<0.0001$, $F=10.35$, $n=6$)	decrease* 100% ($P<0.0001$, $F=108.7$, $n=6$)	decrease* 100% ($P<0.0001$, $F=12.91$, $n=6$)
10 nM	no effect	decrease* 84% ($P<0.0001$, $F=179.6$, $n=6$)	no effect

Note. Values represent maximal changes observed during the experimental; effects lasted for the duration of the experiment unless otherwise stated; p.a., post addition; * denotes statistical significance.

DISCUSSION

In the present study, a novel FaRP, KPSFVRFamide, was isolated and sequenced from an acid ethanol extract of the free-living nematode, *C. elegans*.

Two copies of KPSFVRFamide were found to be encoded on the *flp*-9 gene of *C. elegans*. The encoding gene has been localized to a genomic cluster in the middle of chromosome IV between the genetic markers lin-24 and unc-30 (Fig. 5). PCR experiments on mRNA extracted from eggs, the four larval stages and adult worms, revealed that this gene is selectively expressed in the non-adult stages of the worm. Studies in *Drosophila* have suggested developmentally regulated roles for FaRPs with three neuronal types displaying FMRFamide-like immunoreactivity during larval and/or pupal stages but not the adult (19).

KPSFVRFamide shows considerable structural homology (86%) with the previously identified FaRP, KPNFIRFamide (PF4), which was isolated from the free-living nematode, *P. redivivus* (9). PF4 is very similar to KPNFLRFamide, a peptide predicted to be encoded on the *C. elegans flp*-1 gene. Physiological studies on *A. suum* body wall muscle found that PF4 and KPNFLRFamide had comparable inhibitory activities and potencies, such that the leucine for isoleucine substitution did not confer any apparent differences to the biological activity of these peptides (20).

However, the current study revealed that KPSFVRFamide had no significant effects on tension or contractile parameters of ventral or dorsal somatic body-wall muscle of *A. suum*. Also, in contrast to PF4, it had no effect on the membrane potential of body-wall muscle cells. Clearly, the substitution of serine for asparagine at position 3 and/or valine for isoleucine at position 5 is critical for the biological activity of this peptide.

To examine the potential effects on biological activity of the serine residue at position 3 and the valine residue in position 5, chimeras of KPSFVRFamide and PF4 were synthesized and their physiological actions compared. KPNFVRFamide was found to relax innervated and denervated muscle-strip preparations with a threshold for activity of 1 nM; it also caused a distinct hyperpolarization of somatic body-wall muscle cells (similar to that observed for PF4). Clearly, the asparagine residue in position 3 of the novel *C. elegans* peptide converts it to a peptide with PF4-like activity. In common with PF4, the KPNFVRFamide-induced relaxation of the muscle strip was not found to be mediated by either K^+ or Ca^{2+} ions. Experiments in Cl^--free media indicated that, like PF4 (21), the actions of KPNFVRFamide were dependent on Cl^-.

Although KPSFIRFamide induced a modest muscle cell hyperpolarization in *A. suum*, the extent was >50% less than the hyperpolarization caused by KPNFVRFamide. In contrast to KPNFVRFamide, KPSFIRFamide had no significant effects on *A. suum* muscle-

TABLE 5

Effects of KPSFVRFamide and Chimeric Peptides on *Ascaris suum* Muscle Cell Membrane Potential

Peptide (10 μM)	$\Delta\Sigma_m$ (mV) (at peak)	Replicates
KPSFVRFamide	2.07 ± 2.74	7
KPSFIRFamide	−2.00 ± 1.20	5
KPNFVRFamide	−9.06 ± 1.72	9
Control	0.38 ± 0.38	8

Note. Control: perfusion with artificial perienteric fluid (APF); mV given as means±standard error.

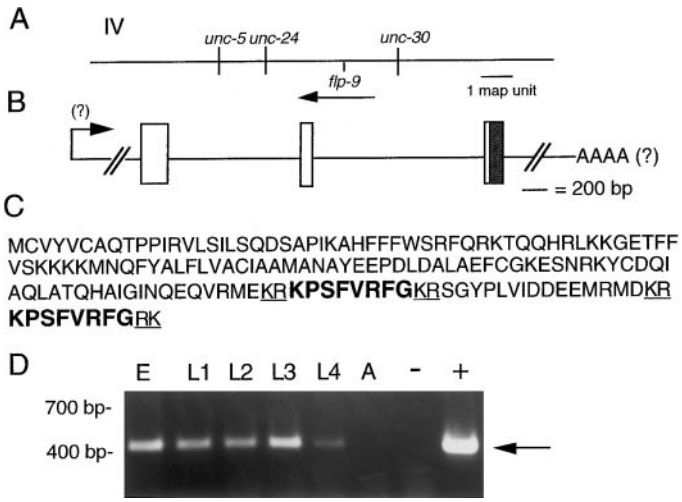

FIG. 5. (A) Chromosomal localization of *flp*-9. *flp*-9 is located on cosmid C36H8, which has been positioned to a gene cluster in the middle of chromosome IV between the genetic markers lin-24 and unc-30. Arrow denotes direction of gene transcription. (B) Genomic organization of *flp*-9. Exons are denoted as boxes, introns as lines. *flp*-9 has at least 3 exons and 2 introns. The start site of transcription (arrow) and the polyadenylation site are unknown. The FaRP-encoding sequence is shaded. (C) Amino acid sequence of the putative *flp*-9 propeptide. Two FaRPs are encoded by the gene and are indicated in bold. Dibasic cleavage sites are underlined. Other non-FMRFamide-related peptides may also be derived from *flp*-9. (D) Expression of *flp*-9. To confirm that *flp*-9 is expressed, RNA isolated from different developmental stages and adults were reverse-transcribed and amplified with *flp*-9 specific primers. Amplification products corresponding to the *flp*-9 transcript (arrow) were detected in cDNA from eggs (E) and larval animals (L1 through L4), but not from adults (A). No product is detected when no cDNA is added (−), amplification from a plasmid containing the *flp*-9 cDNA (+) is shown as a control for the cDNA samples.

strip tension, contraction frequency or amplitude. Why the membrane potential changes caused by KPSFIRFamide were not translated into measurable changes in muscle contractility or tension is unknown. The hyperpolarization may be due to pharmacological effects of high concentrations of this peptide on receptors which are not physiological targets. A similar explanation may hold for the modest effects of KPSFVRFamide on the vagina vera; no peptide with strong homology to KPSFVRFamide has been reported from *A. suum*. It is also possible that the membrane potential changes may have been sub-threshold for the induction of mechanical activity. The lack of biological activity for the parent peptide, KPSFVRFamide, and very minor activity observed for KPSFIRFamide, points to the importance of the serine residue in position 3 of the peptide in distinguishing the biological activity of KPSFVRFamide and KPNFIRFamide.

This study has identified a novel *C. elegans* neuropeptide (KPSFVRFamide), localized it to the *flp*-9 gene on chromosome IV, and shown that although structurally similar to previously identified nematode FaRPs, it does not display the same biological activities on nematode somatic musculature. Studies using chimaeras of KPSFVRFamide revealed that the serine residue in position 3 is responsible for its lack of myoactivity. Also, since there is developmentally regulated expression of the *flp*-9 gene, KPSFVRFamide is implicated in juvenile-specific functions.

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REFERENCES

1. Cowden, C., and Stretton, A. O. W. (1995) *Peptides* **16,** 491–500.
2. Keating, C. D., Holden-dye, L., Thorndyke, M. C., Williams, R. G., Mallett, A., and Walker, R. J. (1995) *Parasitology* **111,** 515–521.
3. Rosoff, M. L., Burglin, T. R., and Li, C. (1992) *J. Neurosci.* **12,** 2356–2361.
4. Marks, N. J., Shaw, C., Maule, A. G., Davis, J. P., Halton, D. W., Verhaert, P., Geary, T. G., and Thompson, D. P. (1995) *Biochem. Biophys. Res. Commun.* **217,** 845–851.
5. Marks, N. J., Maule, A. G., Geary, T. G., Thompson, D. P., Davis, J..P., Halton, D. W., Verhaert, P., and Shaw, C. (1997) *Biochem. Biophys. Res. Commun.* **231,** 591–595.
6. Marks, N. J., Maule, A. G., Geary, T. G., Thompson, D. P., Li, C., Halton, D. W., and Shaw, C. (1998) *Biochem. Biophys. Res. Commun.* (in press).
7. Geary, T. G., Price, D. A., Bowman, J. W., Winterrowd, C. A., MacKenzie, C. D., Garrison, R. D., Williams, J. F., and Friedman, A. R. (1992) *Peptides* **13,** 209–214.
8. Maule, A. G., Shaw, C., Bowman, J. W., Halton, D. W., Thompson, D. P., Geary, T. G., and Thim, L. (1994) *Biochem. Biophys. Res. Commun.* **200,** 973–980.
9. Maule, A. G.; Shaw, C., Bowman, J. W., Halton, D. W., Thompson, D. P., Geary, T. G., Kubiak, T. M., Martin, R. A., and Thim, L. (1995a) *Peptides* **16,** 87–93.
10. Nelson, L. S., Kim, K., Memmott, J. E., and Li, C. (1998) *Mol. Br. Res.* **58,** 103–111.
11. Maule, A. G., Geary, T. G., Bowman, J. W., Shaw, C., Halton, D. W., and Thompson, D. P. (1996) *Parasitol. Today* **12,** 351–357.
12. Gbewonoyo, K., Rohrer, S. P., Lister, L., Burgess, B., Cully, D., and Buckland, B. (1985). *Bio/Technology* **12,** 51–54.
13. Fetterer, R. H., Pax, R. A., and Bennett, J. L. (1977) *Expl. Parasitol.* **43,** 286–294.
14. Marks, N. J., Johnson, S. S., Maule, A. G., Halton, D. W., Shaw, C., Geary, T. G., Moore, S., and Thompson, D. P. (1996) *Parasitology* **113,** 393–401.
15. Fellowes, R. A., Maule, A. G., Marks, N. J., Geary, T. G., Thompson, D. P., Shaw, C., and Halton, D. W. (1998) *Parasitology* **116,** 277–287.
16. Bowman, J. W., Winterrowd, C. A., Friedman, A. R., Thompson, D. P., Klein, R. D., Davis, J. P., Maule, A. G., Blair, K. L., and Geary, T. G (1995) *J. Neurophys.* 74, 1880–1888.

17. Davis, R. E., and Stretton, A. O. W. (1989) *J. Neurosci.* **9,** 403–414.
18. McCormick, J., and Nichols, R. (1993). *J. Comp. Neurol.* **338,** 279–288.
19. Wood, W. B. (1988) Cold Spring Harbour Laboratory pp. 603–604.
20. Kubiak, T., Maule, A. G., Marks, N. J., Martin, R. A., and Wiest, J. R. (1996). *Peptides* **17,** 1267–1277.
21. Maule, A. G., Geary, T. G., Bowman, J. W., Marks, N. J., Blair, K. L., Halton, D. W., Shaw, C.. and Thompson, D. P. (1995b) *Invert. Neurosci.* **1,** 255–265.