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RESEARCH ARTICLE

An unusual myosuppressin from the blood-feeding bug *Rhodnius prolixus*

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SUMMARY

The myosuppressin (MS) gene was cloned from a central nervous system (CNS) cDNA library of the hematophagous insect *Rhodnius prolixus* and is predicted to contain two introns and three exons. The mRNA transcribed from the myosuppressin gene encodes an 88 amino acid prepropeptide, which results in a mature decapeptide after post-translational modification. When compared with the myosuppressins isolated from other insects, the *R. prolixus* myosuppressin has a unique amino acid sequence (pQDIDHVFMRFamide), with isoleucine (I) in position 3 and methionine (M) in position 8. Reverse transcriptase (RT)-PCR shows that *Rhopr-MS* is expressed in the CNS and posterior midgut in *R. prolixus* and immunohistochemistry suggests that an RFamide-like peptide is present in endocrine-like cells in the midgut. Physiological assays using Rhopr-MS indicate that, despite the unusual M at position 8, it still retains myoinhibitory activity, inhibiting the frequency and reducing the amplitude of contractions in the anterior midgut and hindgut, and decreasing heart rate.

Key words: myoinhibitory, immunohistochemistry, midgut, hindgut, heart, contraction.

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INTRODUCTION

Rhodnius prolixus is an important blood-feeding model organism that is extensively used for physiological and endocrinological research. In addition, *R. prolixus*, commonly known as the kissing bug, is a vector of Chagas disease, and is therefore of some medical importance (Rassi et al., 2010). *Rhodnius prolixus* presents some experimental advantages related to its blood-feeding habit. Thus, each unfed instar of *R. prolixus* essentially remains in a state of arrested development until it takes a blood meal. Blood feeding initiates endocrinological and physiological changes that lead to a timed, and predictable, growth and development to the next instar or adult. A number of neurohormones (peptides and amines) are involved in these transitions, some of which are involved in feeding and digestion, while others are involved in growth and development (see Orchard, 2009; Nässel and Winther, 2010)

Recently, Ons and colleagues isolated a number of neuropeptides from the adult *R. prolixus* brain using an off-line nano-LC-MALDI-MS/MS workflow (Ons et al., 2009), and subsequently isolated partial cDNAs for these peptides (Ons et al., 2011). The neuropeptides included FMRF-like peptides (FLPs) that are found extensively throughout the metazoans (see Walker et al., 2009) and share a C-terminal RFamide. Although once referred to as FMRFamide-related peptides, they are now considered to be evolutionarily unrelated (see Orchard and Lange, 2013). One of these groups of FLPs includes the so-called 'myosuppressins' that are only found in insects and crustaceans. In insects, myosuppressins are found in neurons of the central nervous system (CNS) and in midgut endocrine cells, and are therefore brain/gut peptides. Insect myosuppressins have the general conserved sequence: $X^1DVX^4HX^6FLRFamide$ where X^1 can be pQ, P, T or A; X^4 can be D, G or V; and X^6 can be V, S or I (Orchard et al., 2001; Orchard and Lange, 2013; Nygaard et al., 2011). The myosuppressin sequence in *R. prolixus* (Rhopr-MS) isolated by Ons and colleagues (Ons et al. 2009; Ons et al., 2011) has two unique features; the sequence pQDIDHVFMRFamide contains isoleucine (I) in the third position as opposed to the usual valine (V) found in other insects, and also a unique FMRFamide C-terminus and not the usual FLRFamide.

Myosuppressins serve important behavioural and physiological functions in insects (Orchard et al., 2001; Orchard and Lange, 2013) and appear to be one of the core sets of neuropeptides found in all sequenced genomes (see Hauser et al., 2010; Nygaard et al., 2011). For example, they have been shown to inhibit spontaneous contractions of the heart in *Calliphora vomitora* (Duve et al., 1993) and *Protophormia terraenovae* (Angioy et al., 2007), the midgut and oviducts in *Locusta migratoria* (Lange and Orchard, 1998; Lange et al., 1991), and the hindgut in *Leucophaea maderae* (Holman et al., 1986). Myosuppressins also inhibit food intake in *Blattella germanica* (Aguilar et al., 2004), they possess anti-feeding properties in *Spodoptera littoralis* (Vilaplana et al., 2008) and feeding state alters the content of FLPs in endocrine-like cells in the midgut of *L. migratoria* (Lange, 2001).

In light of the unique structural features of Rhopr-MS, we sequenced its cDNA, examined its expression in tissues using reverse transcriptase (RT)-PCR, and also tested its ability to be a myosuppressin on a variety of peripheral tissues.

MATERIALS AND METHODS

Animals

Fifth instar *R. prolixus* Stål 1859 were used from a long-standing colony kept at 25°C and 60% humidity at University of Toronto Mississauga. All insects used were unfed, 4–6 weeks post-emergence, and were previously fed on defibrinated rabbit blood as 4th instars.

Isolation of Rhopr-MS cDNA by screening the 5th instar CNS cDNA library

The cDNA encoding the Rhopr-MS prepropeptide was isolated by screening the 5th instar *R. prolixus* cDNA CNS library (see Paluzzi et al., 2008). The Rhopr-MS amino acid sequence submitted by Ons et al. (Ons et al., 2009) (GenBank ID: ACT98134) was used in a TBLASTN search against the *R. prolixus* preliminary genome assembly database using Geneious Software (Drummond et al., 2010). The predicted *Rhopr-MS*-encoding nucleotide sequence obtained was used to design reverse and forward gene-specific primers (GSPs). Modified 5′ and 3′ RACE (rapid amplification of cDNA ends) were performed using GSPs and CNS cDNA library vector-specific primers (VSPs) (Table 1). The modified RACE procedure allows for amplification of 5′ and 3′ ends of cDNA inserts. For example, the 5′ end was amplified using Myo Rev-1 (GSP) and pDNR-LIB For-2 (VSP) primers and the 3′ end was amplified using Myo For-1 (GSP) and pDNR-LIB Rev-25 (VSP) primers. After the first round of cDNA amplification, all the products were column purified using a PCR Purification Kit (Bio Basic Inc., Markham, ON, Canada). The purified products were then used as templates for the second (nested) PCR. The PCR conditions for the two rounds of PCR were identical, i.e. 5 min initial denaturation at 94°C, 35 cycles of denaturation at 94°C for 15 s each, 35 cycles of annealing at 58°C for 30 s each, 35 cycles of extension at 72°C for 60 s each, and a final extension for 10 min at 72°C. PCR products were separated on a 1% agarose gel stained with ethidium bromide. The amplified products of the three PCR reactions were extracted from the gel and column purified using a Gel Extraction Kit (Bio Basic Inc.). The gel-extracted products were then ligated using the pGEM T-Easy vector system (Promega, Madison, WI, USA). The vectors were subsequently transformed into *E. coli* competent cells using Qiagen PCR Cloning^{plus} Kit (Qiagen, Mississauga, ON, Canada). Positive clones containing the desired inserts were purified, and the *Rhopr-MS* nucleotide sequence determined by the Centre for Applied Genomics at the Hospital for Sick Children (MaRS Centre, Toronto, ON, Canada). The nucleotide sequencing results for the 5′ end, 3′ end, and internal region were aligned using Geneious Pro 5.0.4 software (Drummond et al., 2010).

Sequence analysis of the Rhopr-MS prepropeptide

The presence/location of the signal peptide cleavage sites in the Rhopr-MS prepropeptide sequence was predicted using SignalP 3.0 (Bendtsen et al., 2004). The nucleotide sequence of *Rhopr-MS* obtained by screening the CNS cDNA library was used in a BLAST search against the *R. prolixus* preliminary genome assembly database using Geneious Pro 5.0.4 software (Drummond et al., 2010), which predicted the location and size of the introns in *Rhopr-MS*.

Protein alignment and phylogenetic analysis of Rhopr-MS prepropeptide

The known or predicted myosuppressin precursor amino acid sequences from *R. prolixus* and other insects including *Bombyx mori* (GenBank ID: NP_001166882), *Tribolium castaneum* (GenBank ID: EFA12055), *Apis mellifera* (GenBank ID: ACI90289), *Drosophila melanogaster* (GenBank ID: AAF56283), *Spodoptera littoralis* (GenBank ID: CAO86065), *Anopheles gambiae* (GenBank ID: EAA01711), *Aedes aegypti* (GenBank ID: EAT41022), *Mythimna unipuncta* (GenBank ID: AAL09583), *Blattella germanica* (GenBank ID: CAF04070), *Diploptera punctata* (GenBank ID: AAB39926), *Acyrthosiphon pisum* (GenBank ID: BAH72170), *Acromyrmex echinatior* (GenBank ID: EGI69517) and *Camponotus floridanus* (GenBank ID: EFN71919), as well as from three crustaceans [*Homarus americanus*, GenBank ID: ACX46385; *Procambarus clarkii*, GenBank ID: BAG68789; and *Daphnia pulex* (Dircksen et al., 2011)] were aligned using Clustal W (Larkin et al., 2007). The aligned prepropeptide sequences of identical and similar consensus sequences were denoted with black and grey, respectively, by using the BOXSHADE3.21 server (www.ch.embnet.org/software/BOX_form.html). A phylogenetic tree was created using the protein alignment on Molecular Evolutionary Genetics Analysis (MEGA) Version 4.0.2 software (see Tamura et al., 2007) by using the maximum-likelihood method (see Jones et al., 1992) and is presented along with bootstrap values based on 1000 replicates.

Table 1. Gene-specific and vector-specific primers used for cloning the Rhopr-MS cDNA

	Nucleotide sequence
Gene-specific primers	
Myo For-1	5′-TTCAACGCTATCTCGCAAAG-3′
Myo For-2	5′-TAAACACGTCGATTTGGCTG-3′
Myo Rev-1	5′-ACTCTCTGCCACTTAAATTTTCTTG-3′
Myo Rev-2	5′-TTTTGTTCATCTAGATAAGCCTGC-3′
Vector-specific primers	
pDNR-LIB For-2	5′-ACGGTACCGGACATATGCC-3′
pDNR-LIB Rev-25	5′-GCCAAACGAATGGTCTAGAAAG-3′

Rhopr-MS expression using RT-PCR

To determine the expression of *Rhopr-MS* in different tissues of 5th instar *R. prolixus*, RT-PCR was performed on the CNS, foregut, anterior midgut, posterior midgut, hindgut, Malpighian tubules, testes, ovaries, dorsal vessel/fat bodies/trachea and salivary glands. The tissues were dissected from 5th instar *R. prolixus* in nuclease-free phosphate-buffered saline (PBS) (Sigma-Aldrich, Oakville, ON, Canada) and were stored in RNAlater^{TM} RNA stabilization reagent (Qiagen Inc.) at –20°C. Total RNA was extracted from each of the tissues using SV Total RNA Isolation System (Promega) and quantified using a NanoDrop 2000 UV spectrophotometer (ThermoFisher Scientific, Wilmington, DE, USA). Total RNA (100 ng) was used from each tissue to synthesize cDNA using iScript^{TM} Select cDNA Synthesis Kit (Bio-Rad, Mississauga, ON, Canada), which was used as template to perform PCR. The primers used were Myo For-1 and Myo Rev-1 (Table 1), and the conditions of the thermocycler used to run the PCR were the same as described above for modified RACE experiments. To test the quality of the cDNA template, actin was used as a positive control. The RT-PCR products were separated on a 1% agarose gel stained with ethidium bromide.

Immunohistochemistry and bioassays

Chemicals

Rhopr-MS (purity 93.1%) was purchased from GenScript USA Inc. (Piscataway, NJ, USA). Stock solutions in 10 µl aliquots of 10^{-3} mol l^{-1} Rhopr-MS were made using 10% dimethylsulphoxide (DMSO; Sigma-Aldrich) in ddH_2O, and were stored at –20°C. Further dilutions were made using physiological saline (150 mmol l^{-1} NaCl, 8.6 mmol l^{-1} KCl, 2 mmol l^{-1} $CaCl_2$, 34 mmol l^{-1} glucose, 4 mmol l^{-1} $NaHCO_3$, 8.5 mmol l^{-1} $MgCl_2$, 5.0 mmol l^{-1} Hepes pH 7.0) as suggested by Lane et al. (Lane et al., 1975). The maximum concentration of DMSO used in the assays was 0.001% and control experiments using this concentration showed no effect on the tested tissues.

Immunohistochemistry

The dorsal cuticle of the head capsule and the ventral abdominal cuticle of 5th instar *R. prolixus* were dissected under saline, exposing the digestive tract and CNS. Fixative consisting of 2% paraformaldehyde in Millonig's buffer (0.13 mol l^{-1} $NaH_2PO_4 \cdot H_2O$, 0.1 mol l^{-1} NaOH, 1.2% glucose, 0.3 mmol l^{-1} $CaCl_2$; pH 7) was applied for 1 h at room temperature. Tissues were then washed with PBS (containing 0.9% NaCl, pH 7.2) for 1 h with rinses every 5 min. The digestive tract and CNS were removed and incubated for 1 h at room temperature in 4% Triton X-100 made up in PBS containing 10% normal goat serum (NGS) and 2% bovine serum albumin (BSA). Tissues were then washed with PBS for 1 h with rinses every 10 min and were subsequently incubated on a shaker for 48 h at 4°C in 1:1000 rabbit anti-FMRFamide antiserum (INCSTAR, Stillwater, MN, USA) made up in PBS containing 0.4% Triton X-100, 2% BSA and 2% NGS. Following incubations, the tissues were washed in PBS for 2 h with rinses every 10 min, and then incubated for 18 h at 4°C in secondary antibody solution of purified goat anti-rabbit conjugated to Cy3 (diluted 1:600), containing 10% NGS in PBS. Tissues were then washed with PBS for 8 h with rinses every 10 min. Tissues were mounted on microscope slides in 100% glycerol. FMRFamide-like immunoreactivity was observed with a Zeiss confocal laser microscope (Carl Zeiss, Jena, Germany) and Zen 2009 viewing software (Zeiss). Controls were run whereby the primary antiserum was preabsorbed with 10^{-5} mol l^{-1} synthetic Rhopr-MS prior to use. All staining of cell bodies and processes was eliminated using preabsorbed antiserum

Midgut and hindgut assays

Contraction assays were performed on isolated tissues from 5th instar *R. prolixus*. A fine silk thread was tied at the anterior end of the anterior midgut, and another at the most posterior end of the anterior midgut. The most posterior end was tied to a minuten pin secured to a well in a Sylgard-coated Petri dish containing 200 µl saline, and the silk thread attached to the anterior end was attached to a force transducer (Aksjeselskapet Mikro-elektronikk, Horten, Norway). For hindgut assays, the posterior end of the hindgut was dissected and kept attached to a small piece of cuticle surrounding the anus. The cuticle and attached hindgut was then secured to a Sylgard-coated Petri dish (in a well containing 200 µl saline), while the anterior end of the hindgut was tied with a fine silk thread and attached to a force transducer. Anterior midgut and hindgut muscle contractions were monitored on a linear flatbed chart recorder through an amplifier. Rhopr-MS was bath applied to the tissues by removing half of the saline present in the well containing the tissue, and simultaneously replacing it with an equal volume of the test solution at twice the desired, final, concentration.

Heart rate assays

The ventral abdominal cuticle and visceral tissues were removed from 5th instar *R. prolixus*, leaving the dorsal vessel exposed. Semi-intact preparations of the abdominal region of the dorsal vessel and surrounding dorsal cuticle were secured in a dish. Heart rate was measured in saline and in various doses of Rhopr-MS using electrodes connected to an impedance converter (UFI model 2991, Morro Bay, CA, USA). Electrodes were placed on either side of the heart between the sixth and seventh abdominal segments. Preparations were maintained with 50 µl saline, and test solutions were added by removing all saline, and subsequently adding 50 µl of the desired concentration of peptide.

RESULTS

Isolation of the *Rhopr-MS* cDNA

The modified RACE experiments resulted in cloning of a 437 bp long *Rhopr-MS* cDNA sequence that encodes a prepropeptide of 88 amino acid residues (Fig. 1). Comparison of the nucleotide sequence of *Rhopr-MS* cDNA with the genomic sequence reveals the presence of two introns and three exons in *Rhopr-MS*. The first intron is located between nucleotides 120 and 121 (Fig. 1) and is at least 132,502 bp in length. The second intron is located between nucleotides 295 and 296 (Fig. 1) and is 1479 bp in length.

The putative signal peptide sequence is observed within Rhopr-MS prepropeptide and its cleavage site is predicted to be between amino acid residues 16 and 17 using the neural networks (NN) and hidden Markov model (HMM) prediction available on SignalP 3.0 (see Bendsten et al., 2004) (Fig. 1). The mature Rhopr-MS peptide, QDIDHVFMRFG, is released after cleaving at dibasic (KR) and tribasic (RRR) cleavage sites (Fig. 1). The mature Rhopr-MS is amidated and the N-terminal glutamic acid residue forms a pyroglutamic acid during post-translational modification, resulting in the sequence pQDIDHVFMRFamide, as determined by Ons et al. (Ons et al., 2011). We have also confirmed the amino acid sequence using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) of CNS extracts from 5th instar *R. prolixus*. A stop codon (TAA) was observed immediately after the tribasic cleavage site. One putative poly-A tail signal was observed at the very 3′ end of the sequence.

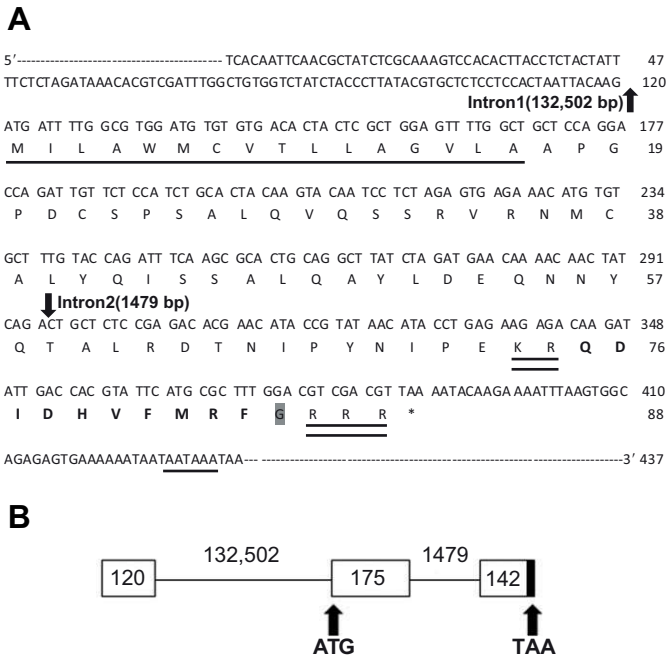

Fig. 1. (A) Nucleotide cDNA sequence and deduced amino acid prepropeptide of the *Rhodnius prolixus* myosuppressin gene (*Rhopr-MS*; GenBank ID: JN830619). The coding region starts at the nucleotide sequence ATG and stops at the sequence TAA (denoted by an asterisk). The highly predicted signal peptide is underlined and dibasic (KR) and tribasic (RRR) cleavage sites are double underline. The mature Rhopr-MS is in bold and the glycine residue required for amidation is highlighted in grey. Two introns in *Rhopr-MS* were predicted and are indicated by the black arrows. (B) Schematic diagram of *Rhopr-MS* showing the location and lengths of introns (lines) and exons (boxes). The numbers refer to the size of the exons and introns in base pairs. The start site (ATG) of the prepropeptide and the stop codon (TAA) are also shown in this figure. The black bar in exon 3 represents the mature Rhopr-MS decapeptide.

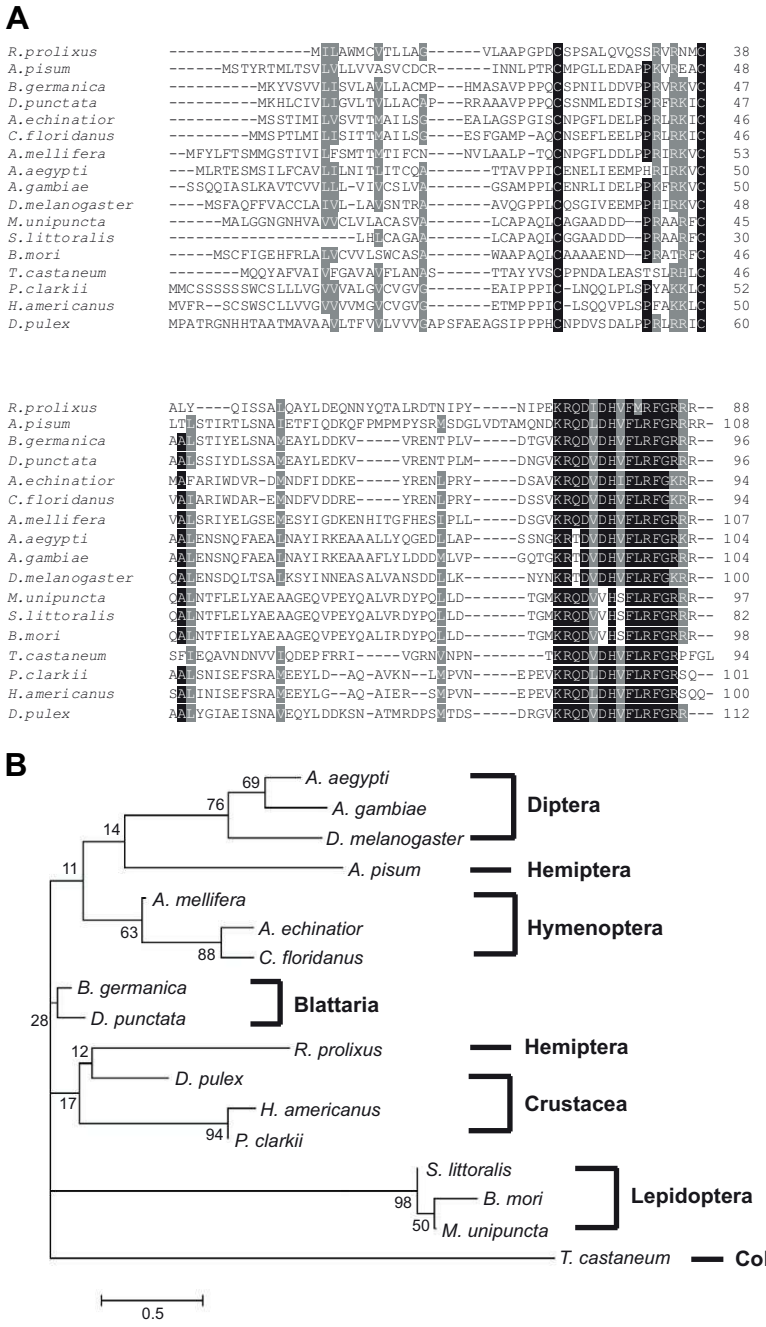

Fig. 2. (A) Protein alignment and phylogenetic analysis of known or predicted (from genome) myosuppressin precursor sequences from 14 different insect species and three crustacean species. Sequences were aligned with ClustalW. Following the 80% majority rule, identical amino acids are highlighted in black and similar amino acids are highlighted in grey. The following species are represented on the alignment and phylogenetic tree: *Rhodnius prolixus*, *Acyrthosiphon pisum*, *Blattella germanica*, *Diploptera punctata*, *Acromyrmex echinatior*, *Camponotus floridanus*, *Apis mellifera*, *Aedes aegypti*, *Anopheles gambiae*, *Drosophila melanogaster*, *Mythimna unipuncta*, *Spodoptera littoralis*, *Bombyx mori*, *Tribolium castaneum*, *Procambarus clarkii*, *Homarus americanus* and *Daphnia pulex*. (B) Phylogenetic relationship between the myosuppressin prepropeptide sequences of different species. The tree was created using the maximum likelihood method based on Jones et al. (Jones et al., 1992) with frequency model. The numbers at the nodes represent percentage support in 1000 bootstrap replicates. All positions containing gaps and missing data were eliminated. The tree is drawn to scale.

Protein alignment and phylogenetic analysis

Protein alignment was performed using ClustalW Version 2.0 (see Larkin et al., 2007) with myosuppressin prepropeptide sequences (either cloned or predicted from the genome) of 14 different insects, and three species of crustaceans. The protein alignment reveals a highly conserved sequence of the mature myosuppressin in all species of insects and crustaceans included in the alignment (Fig. 2A). However, the crustaceans, *P. clarkii* and *H. americanus*, have a leucine (L) residue in the third amino acid position, whereas *D. pulex* and almost all the other insects examined have a V residue. Interestingly, *R. prolixus* and *A. pisum* (both hemipterans) have an I and L residue, respectively, in the third amino acid position (Fig. 2A). Moreover, *R. prolixus* is the only species among insects and crustaceans known to have a unique FMRFamide C-terminus (Fig. 2A). All other species have an FLRFamide C-terminus (Fig. 2A).

The phylogenetic tree shows that *R. prolixus* is separated from the hemipteran *A. pisum* with respect to the myosuppressin prepropeptide, and appears to group with Crustacea. Also, *A. pisum* and the Diptera, *A. aegypti*, *A. gambia* and *D. melanogaster*, form a monophyletic group (Fig. 2B).

Analysis of *Rhopr-MS* expression through RT-PCR

Total RNA was extracted from different tissues and used to synthesize cDNA. RT-PCR was performed using cDNA from different tissues as the template. The results show that *Rhopr-MS* is expressed in only two of the tissues examined, the CNS and posterior midgut of *R. prolixus* (Fig. 3). Actin was used as a control to test the quality of the cDNA.

Immunohistochemistry

FMRFamide-like immunoreactivity is distributed extensively in the neurons of the CNS, in processes associated with the hindgut of *R.*

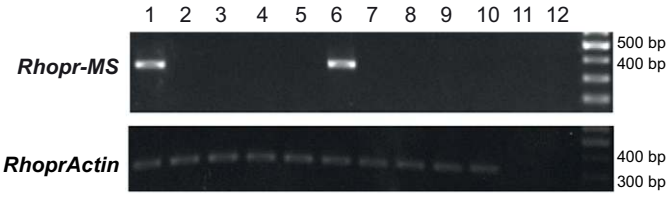

Fig. 3. The expression of *Rhopr-MS* mRNA in different tissues of 5th instar *R. prolixus*. Expression is seen in the central nervous system (CNS) and posterior midgut. Myo For-1 and Myo Rev-1 (see Table 1) primers were used to amplify the *Rhopr-MS* cDNA sequence in different tissues and the expected product size was 410 bp. The primers used for positive control (actin) are expected to give a product of 320 bp. Two negative controls were performed, one in which cDNA was synthesized without adding any RNA, and a second that contained no cDNA template. The numbers indicate the following tissues: (1) CNS, (2) testes, (3) ovaries, (4) foregut, (5) anterior midgut, (6) posterior midgut, (7) hindgut, (8) dorsal vessel/fat bodies/trachea, (9) Malpighian tubules, (10) salivary glands, (11) no template, (12) negative control. Tissues from at least 10 insects were dissected and pooled. The results shown are of three independent biological replicates.

prolixus, and in processes over the foregut and hindgut (Tsang and Orchard, 1991). Closer examination of the anterior and posterior midgut of 5th instar *R. prolixus* for FMRFamide-like immunoreactivity revealed FMRFamide-like immunoreactive endocrine-like cells (Fig. 4). These endocrine-like cells had apical processes that extended towards the midgut lumen.

Physiological effects of Rhopr-MS on peripheral tissues

Rhopr-MS decreased the frequency and amplitude of spontaneous phasic contractions of the anterior midgut (Fig. 5) and hindgut (Fig. 6) in a dose-dependent manner. The threshold lay between 10^{-12} and 10^{-11} mol l^{-1} Rhopr-MS. Maximal inhibition was observed at 10^{-9} mol l^{-1} Rhopr-MS for the anterior midgut and 5×10^{-8} mol l^{-1} Rhopr-MS for the hindgut, and resulted in a cessation of contractions. Rhopr-MS also decreased heart rate (Fig. 7), with the threshold between 10^{-11} and 10^{-10} mol l^{-1}, and total inhibition of heartbeat occurred at 10^{-7} mol l^{-1}. The EC_{50} for inhibition of contraction for hindgut was 1.3×10^{-9} mol l^{-1} and for heart it was 6×10^{-9} mol l^{-1}.

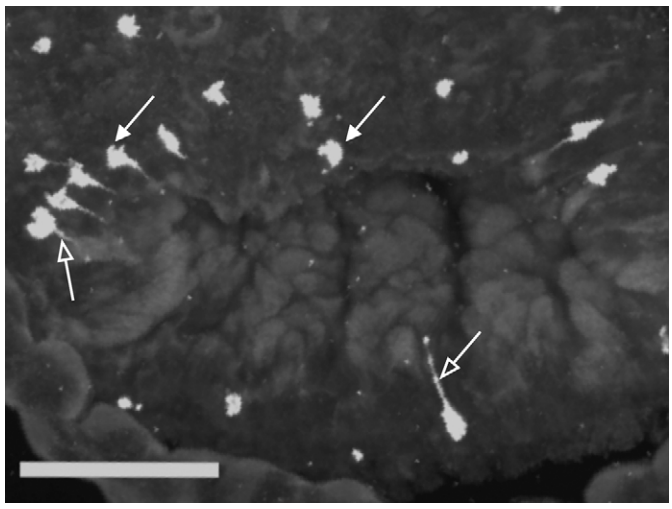

Fig. 4. FMRF-amide like immunoreactivity is associated with endocrine-like cells in the midgut of 5th instar *R. prolixus*. Endocrine-like cells (filled arrows) within the anterior midgut are shown in the stacked confocal image. These endocrine-like cells have apical processes (open arrows) that extend towards the lumen. Scale bar 100 μm, *N*=10.

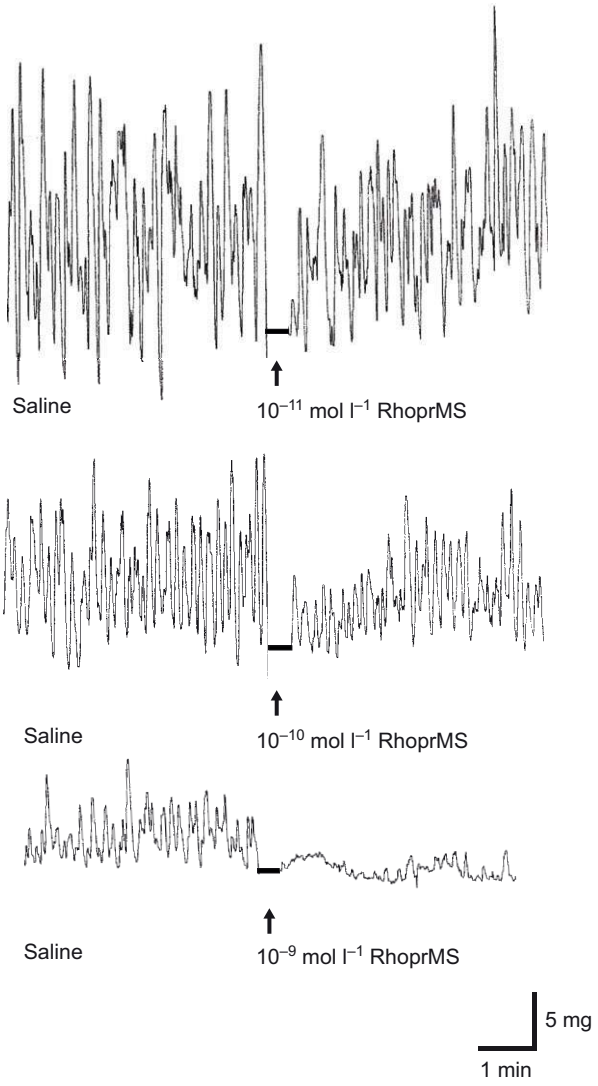

Fig. 5. The effects of Rhopr-MS on phasic contractions of the anterior midgut of 5th instar *R. prolixus*. The sample traces illustrate that Rhopr-MS inhibits the frequency and amplitude of anterior midgut contractions. Arrows indicate the addition of Rhopr-MS. Similar results were obtained from six preparations.

DISCUSSION

Since the discovery of the molluscan cardioacceleratory tetrapeptide FMRFamide (Price and Greenberg, 1977), a variety of neuropeptides that share the C-terminal RFamide have been characterized in insects. These FLPs fall into several families of peptides, one of which is the myosuppressins (see Orchard and Lange, 2013; Nässel and Winther, 2010). Insect myosuppressins have a fairly conserved sequence, $X^1DVX^4HX^6FLRFamide$ (where X^1 is pQ, Q, P, T or A; X^4 is D, G or V; and X^6 is V, S or I).

We have partially cloned the myosuppressin cDNA in *R. prolixus*, resulting in a 437 bp nucleotide sequence that codes for an 88 amino acid prepropeptide. The amino acid sequence of the open reading

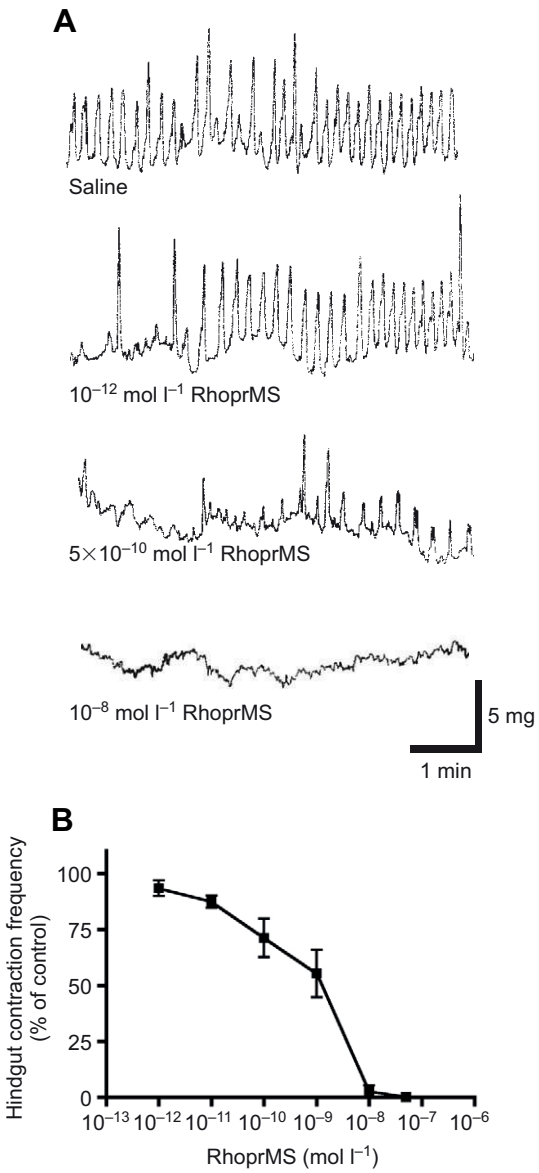

Fig. 6. The effect of Rhopr-MS on phasic contractions of the hindgut of 5th instar *R. prolixus*. (A) Sample traces illustrate that Rhopr-MS inhibits the frequency and amplitude of hindgut contractions. (B) Dose–response curve shows that Rhopr-MS leads to a dose-dependent decrease in the frequency of contraction as a percentage of the saline control. Data are means ± s.e.m., *N*=5.

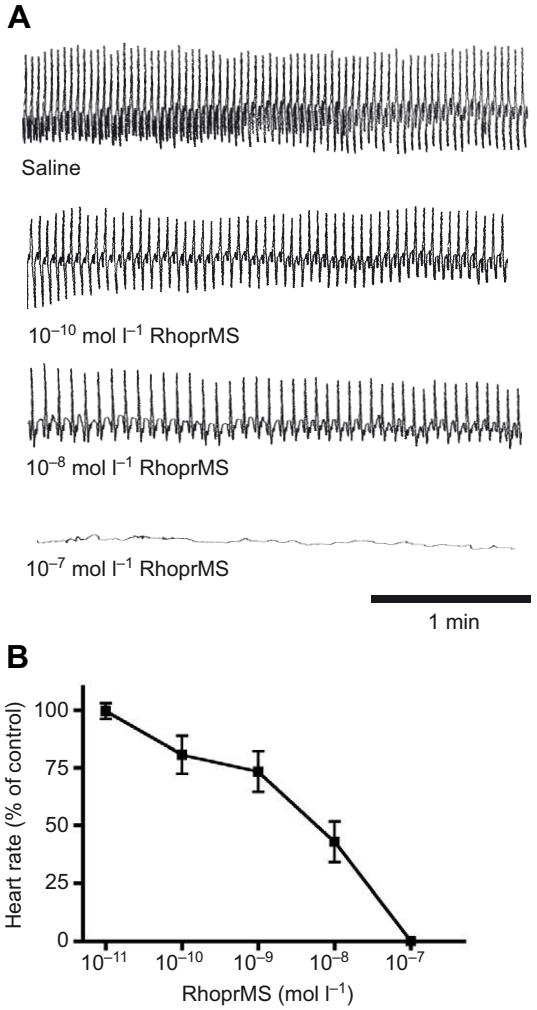

Fig. 7. The effect of Rhopr-MS on the heart rate of 5th instar *R. prolixus*. (A) Sample traces illustrate that Rhopr-MS inhibits the frequency of heart contractions. (B) Dose–response curve shows that increasing the concentration of Rhopr-MS leads to a dose-dependent decrease in heart rate relative to saline controls. Data are means ± s.e.m., *N*=6.

frame is identical to that identified previously (Ons et al., 2011; Sterkel et al., 2011). After post-translational modifications, a mature decapeptide is produced, which has the sequence: pQDIDHVFMRFamide. The myosuppressin in *R. prolixus* has some unique features wherein X^3 is I and X^8 is M (methionine). Interestingly, other extended FMRFamides are also found in *R. prolixus* (Ons et al., 2011), resulting in the only FMRFamide to be found outside of the dipterans and the only myosuppressin without an FLRFamide motif. The myosuppressin cDNA was recently cloned in another hemipteran, the pea aphid, *A. pisum*, and it contains L in the third amino acid position (Shigenobu et al., 2010). The *R. prolixus* myosuppressin is also unique from other insect myosuppresins in that the amino acid in position three is I whereas V is conserved in most insects. Interestingly, in *H. americanus* and *P. clarkii*, the third position is occupied by the amino acid L similar to that seen in *A. pisum*.

Ons et al. (Ons et al., 2011), using bioinformatic tools and manual analysis, predicted that there are four peptides encoded in the myosuppresin prepropeptide with three of these confirmed by MALDI-TOF MS/MS; QDIDHVFMRFamide, pQDIDHVFMRFamide and pQDIDHVF(M-OH)RFamide. The latter two myosuppressins were confirmed using nano-LC and ESI-Orbi-Trap MS/MS (Sterkel et al., 2011) and are post-translationally modified versions of QDIDHVFMRFamide. The presence of pQ (pyro-glutamate) is common in myosuppressins of insects (see Nässel and Winther, 2010; Orchard and Lange, 2013), but the addition of a hydroxide group on the methionine has not been reported for myosuppresins and could either be a rare form of this peptide or a result of the procedure used to isolate these peptides during mass spectrometry (Ons et al., 2010). We have also confirmed the presence of pQDIDHVFMRFamide by MALDI-TOF MS from CNS extracts of *R. prolixus*. More experiments must be performed to confirm the existence and quantity of the various myosuppressins and their presence in a variety of tissues in *R. prolixus*.

The RT-PCR results show that *Rhopr-MS* mRNA is expressed in the CNS and posterior midgut. The *Rhopr-MS* cDNA was isolated and sequenced using the CNS cDNA library, which also indicates its presence in the CNS. Recently, *Rhopr-MS* expression was also observed in the posterior midgut of *R. prolixus* but not in the crop (anterior midgut) or hindgut (Sterkel et al., 2011). *In situ* hybridization in *M. sexta* and *D. punctata* reveals myosuppressin expression in endocrine cells of the posterior midgut (Fusé et al., 1998; Lu et al., 2002).

Immunohistochemistry using an anti-RFamide antibody in *R. prolixus* reveals immunostained processes projecting to the hindgut (Tsang and Orchard, 1991) and also positively stained endocrine-like cells throughout the entire midgut (this study). These endocrine-like cells appear to be of the open type with processes extending from the cell body towards the lumen. The presence of myosuppressin in endocrine-like cells of the midgut suggests that myosuppressin plays a role in feeding/digestion. A number of studies have now shown a role of FLPs in feeding-related behaviour. Vilaplana et al. have shown that myosuppressins inhibit food intake in *S. littoralis* (Vilaplana et al., 2008). Moreover, in *B. germanica*, mRNA expression levels of myosuppressin slightly decrease during maximum food intake (Vilaplana et al., 2004). Myosuppressins also stimulate the release of the digestive enzyme α-amylase in the red palm weevil (beetle), *Rynchphorus ferrugineus* (Nachman et al., 1997). Therefore, it appears that myosuppressin may play a role in digestion, acting as a neurotransmitter directly delivered to the hindgut and midgut tissue or locally released from endocrine-like cells. The presence of FLPs in the haemolymph of *R. prolixus* indicates that it might be acting as a neurohormone (Elia et al., 1993). In *R. prolixus*, myosuppressin expression is not present in the salivary glands; however, FLPs in the nerve supply to the salivary glands indicate that myosuppressin may act as a neurotransmitter at this tissue (Orchard and Te Brugge, 2002).

Myosuppressins, as their name implies, inhibit contractions of visceral and cardiac muscle (see Orchard et al., 2001; Nässel and Winther, 2010; Orchard and Lange, 2013), but in some insect species they have also been shown to enhance the force of neurally evoked contractions of skeletal muscle (Robb and Evans, 1994). Myosuppressins also stimulate enzyme secretion and inhibit short-circuit current in the midgut, and inhibit the release of adipokinetic hormones (see Orchard et al., 2001; Orchard and Lange, 2013). Injection of the cockroach myosuppressin into *B. germanica* inhibits food intake, with food accumulating in the foregut (Vilaplana et al., 2004). In *R. prolixus*, Rhopr-MS was very effective in inhibiting the frequency and amplitude of phasic contractions of the anterior midgut (crop) and hindgut, as well as decreasing heart rate, with an EC_{50} of 1.3×10^{-9} mol l^{-1} for the hindgut and 6×10^{-9} mol l^{-1} for heart rate. In all three tissues, Rhopr-MS was capable of eliminating all phasic contractions.

It is interesting that even though the amino acid sequence of Rhopr-MS has some unique features it still retains inhibitory activity over visceral and cardiac muscle contraction. An *in vitro* binding assay has been used to characterize the putative receptors for *L. migratoria* myosuppressin associated with the oviduct and CNS of *L. migratoria* (Wang et al., 1995a) (see also Orchard et al., 2001; Orchard and Lange, 2013), and structure–activity relationships have been examined in *L. maderae* hindgut and *L. migratoria* oviduct (see Orchard et al., 2001). In the hindgut, VFLRFamide appears to be the active core for inhibitory biological activity. In the oviduct, the amide is crucial for both binding with the receptor and inhibitory biological activity. Interestingly, VFLRFamide, which produces stimulatory responses on the locust oviduct, is the minimum sequence required for receptor binding whereas HVFLRFamide is the minimum sequence for comparable inhibitory biological activity (Wang et al., 1995a). Therefore, in the locust oviducts, inhibitory and stimulatory peptides share a single receptor but are capable of producing opposite muscle responses, *via* two different intracellular signalling systems, as a result of differences in the activation sites. Structure/activity experiments examining binding affinity and physiological activity of HVFLRFamide analogues indicate that replacement of the L with a similar amino acid such as V resulted in an analogue that bound to the receptor with reduced potency but was still capable of inhibiting contractions of locust oviducts (Wang et al., 1995b). Replacement with a dissimilar amino acid such as D (aspartate) resulted in reduced binding and no biological activity. The present data using Rhopr-MS indicate that myosuppressins can also tolerate a change to M in the 8th position and still bind to the receptor and have inhibitory activity.

The isolation and cloning of *Rhopr-MS* cDNA and the demonstration that the peptide retains myosuppressin activity even though it has a unique amino acid sequence, is of some interest. The myosuppressins are considered to be one of the core neuropeptides, with their genes present in all insect genomes (see Hauser et al., 2010; Nygaard et al., 2011). As such, they must control fundamental processes required by all insects. The physiological and behavioural role of Rhopr-MS needs to be examined in detail to better understand the significance of this peptide in the medically important bug *R. prolixus*.

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