



Research Paper

Expression and functional characterization of tachykinin-related peptides in the blood-feeding bug, *Rhodnius prolixus*



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ABSTRACT

Tachykinins (tachykinin-related peptides, TRPs) are multifunctional neuropeptides that have widespread distribution in the central nervous system (CNS) and in the gastrointestinal tract of many insects, and most have been shown to stimulate contractions of visceral muscles. Invertebrate TRPs carry a characteristic conserved C-terminal pentapeptide (FXGXR-amide) and most of them share some amino acid sequence similarities (approx. 45%) with the vertebrate and mammalian tachykinin family. We have functionally characterized the tachykinins in *R. prolixus* (Rhopr-TKs) and partially cloned the transcript that encodes for the peptide precursor. The transcript encodes 8 Rhopr-TKs, 7 of which are unique with Rhopr-TK 5 having 2 copies. The spatial distribution analysis of the Rhopr-TK transcript indicates that the highest expression levels are in the CNS, but transcript expression is also associated with salivary glands, fat body, dorsal vessel, and the various gut compartments. Rhopr-TK 1, 2 and 5 significantly increase the frequency and amplitude of peristaltic contractions of the salivary glands. Hindgut muscle also displayed a dose-dependent increase in basal tonus in response to Rhopr-TK1, 2 and 5. TK-like immunoreactivity was seen in a small group of processes that are situated on the lateral margins of the hindgut. Interestingly, kinin-like immunoreactivity is seen in immunoreactive processes on the lateral margin of the hindgut as well as fine processes covering the entire hindgut. Co-localization studies show that TK-like staining is always co-localized with kinin-like immunoreactivity, whereas kinin-like staining is seen in the fine processes that are devoid of TK-like immunoreactivity indicating that TKs are most likely released together with kinins to act on the hindgut. Rhopr-Kinin 2 is a potent stimulator of hindgut muscle contraction in *R. prolixus*. Addition of Rhopr-Kinin 2 and Rhopr-TK 2 to the hindgut leads to a contraction that was additive of the effects of Rhopr-Kinin 2 and Rhopr-TK 2 alone.

1. Introduction

Neuropeptides play fundamental roles in the control of development, behaviour, reproduction, feeding, and many other physiological processes in the blood-feeding hemipteran, *Rhodnius prolixus* [1,23,24,27,28,36]. Tachykinins (tachykinin-related peptides, TRPs) are one of the largest families of peptides that are present in all animal species, from invertebrates to mammals [18,21]. Tachykinins are multifunctional neuropeptides widely distributed in the central nervous system (CNS), the gastrointestinal tract, and many other tissues of several arthropods, annelids, mollusks and cnidarians [21,32,33,46,48,50].

TRPs act as neurotransmitters and neuromodulators, but also act as autocrine, paracrine, and endocrine regulators in peripheral tissues [4,18,21,37,38]. *Locusta migratoria* TKs (Lom-TKs) were the first invertebrate TRPs to be isolated, and were extracted from the CNS [34,35]. Stimulatory effects of Lom-TKs were observed on hindgut

muscle contractions in *L. migratoria*, *R. prolixus* and in the cockroach *Leucophaea maderae* [12,34,49]. Lom-TKs also stimulate dose-dependent increases in contractions of the locust oviduct [12] and stimulate the release of other neuropeptides, such as adipokinetic hormone from the corpus cardiacum [20]. In addition, in *Drosophila melanogaster*, TRPs (DTK-1 to 5) play a role in regulating the production of an insulin-like peptide (DILP5) in the Malpighian tubules [41]. The disruption of DTK signalling, using RNAi, was found to reduce the sensitivity of *D. melanogaster* larvae to odorants [46,51]. Recent work on the cockroach *Periplaneta americana* and on *D. melanogaster* reveals that nutrients can upregulate the secretion of TRPs by midgut endocrine cells, influencing the activities of digestive enzymes such as amylases, proteases and lipases, and regulating intestinal lipid metabolism [16,42]. Recently, a neuropeptide named Natalisin (C-terminus consensus sequence of FXXXRamide), which contains a C-terminal motif that is closely related to that of the TRPs, was described in several species [10]. This neuropeptide is expressed in neurons in the brain and regulates mating

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behaviour in *D. melanogaster* and fecundity in *Tribolium castaneum* and *Bombyx mori* [10].

Many invertebrate TRPs possess a characteristic conserved C-terminal pentapeptide FXGXRamide, which is similar to vertebrate tachykinins that end with FXGLMamide, as found in Substance P and Neurokinin A and B [11,17,21,32,33]. This latter motif is also present in Sialokinin I and II, isolated from the salivary glands of the yellow fever mosquito, *Aedes aegypti*, and which act as vasodilators in mammals [6]. Eledoisin from *Eledone moschata*, and OctTK I–II from *Octopus vulgaris* are also characterized by a common C-terminal sequence FXGLMamide [21].

Tachykinin-related peptides have been intensively studied in *D. melanogaster*, *L. migratoria* and the cockroaches *L. maderae* and *P. americana*; however, little is known about the role of Rhopr-TKs in *R. prolixus*. *Rhodnius prolixus* is a blood-gorging hemipteran, and one of the most important vectors of Chagas disease [8]. Successful and efficient gorging, of up to 10X their initial body weight in blood, requires the injection of saliva into the host, which contains many physiologically active compounds that facilitate the blood-feeding process [30]. *Rhodnius prolixus* that have their salivary glands surgically removed ingest smaller amounts of blood from a living host [30]. In a previous study on unfed 5th instar *R. prolixus*, TK-like immunoreactive neurons and processes were found throughout the CNS and immunoreactive processes were seen over the hindgut. Two *L. migratoria* TKs, Lom-TK I and II, were found to increase basal tonus and frequency and amplitude of contractions of the hindgut, suggesting that a tachykinin-like peptide is likely released from processes at the hindgut to control contraction [13].

In the present study, we have cloned the cDNA sequence of the *R. prolixus* tachykinin transcript open reading frame (ORF), which had been previously identified in the genome [22] and quantitative PCR (qPCR) was used to study its spatial distribution in *R. prolixus*. To identify possible functions for Rhopr-TKs in *R. prolixus* the effects of 3 Rhopr-TKs on hindgut and salivary gland muscle contraction was examined.

2. Materials and methods

2.1. Insects

Fifth-instar *R. prolixus* were obtained from a long-standing colony at the University of Toronto Mississauga. Insects were reared at 50% humidity, 25 °C, and fed once during each developmental stage with defibrinated rabbit blood (Cedarlane Laboratories Inc., Burlington, ON, Canada) using a latex membrane-based artificial feeding system. Insects were dissected under *R. prolixus* physiological saline (NaCl 150 mM, KCl 8.6 mM, CaCl₂ 2.0 mM, MgCl₂ 8.5 mM, NaHCO₃ 4.0 mM, glucose 34.0 mM, HEPES 5.0 mM, pH 7.0).

2.2. Chemicals

Anti-Lom-TK-1 and anti-Lem-K-1 antisera were provided by Dr. D. Nässel (Stockholm University, Stockholm, Sweden). Goat cyanine dye 3 (Cy3) anti-rabbit (IgG) secondary antibody was purchased from Jackson Immuno-Research Laboratories, Inc. (West Grove, PA, USA). *Rhodnius prolixus* tachykinin-related peptides (Rhopr-TK1 pQERRAMGFVGMRamide, Rhopr-TK2 APSTMGFQGVRamide and Rhopr-TK5 APASGFFGMRamide), and Rhopr-Kinin 2 (AKFSSWGamide) were custom synthesized by GenScript (Piscataway, NJ, USA) at > 95% purity. All peptides were reconstituted in double distilled water into 10^{−3} M stock solutions, and stored at −20 °C until the working solutions were prepared using physiological saline. All other chemicals were purchased from Sigma-Aldrich Corporation (Missouri, USA).

2.3. Cloning of the Rhopr-TK transcript

The CNS was dissected from 3 groups of fifteen 5th instars and total RNA was extracted using the PureLink RNA mini kit (Life Technologies Corporation, Carlsbad, CA, USA), and quantified using a NanoDrop System (ND-1000 Thermo Scientific, Burlington, ON, Canada). Subsequently, the cDNA was synthesized using iScript Reverse Transcription Supermix (Bio-Rad, Mississauga, ON, Canada), and an aliquot of 200 ng of cDNA was used as a template to perform the subsequent PCR.

To amplify the transcript, forward (5' TTCGCTCACACCCGTCCTCA 3') and reverse (5' CGGTTTATATATCGTATAG 3') primers were designed based on the Rhopr-TK predicted sequence (GenBank: GQ162785.1) [22]. The PCR products were analysed on 1.5% agarose gels stained with RedSafe (Frogga Bio, Toronto, ON, Canada) and then column purified and cloned using the pGEM-T Easy Vector system (Promega, Madison, WI, USA). The clones expressing the desired products were column purified (Bio Basic Canada INC., Markham, ON, Canada) and the sequences were determined at the Centre for Applied Genomics at the Hospital for Sick Children (MaRS Centre, Toronto, ON, Canada). For base accuracy sequencing of Rhopr-TK, the cloning was repeated three times and at least 5 clones were sequenced.

2.4. Tachykinin-related peptide precursor alignment and phylogenetic analysis

The deduced amino acid sequence of the Rhopr-TK precursor was aligned with seventeen insect tachykinin precursors (*Bombus impatiens* XP_003490924.1, *B. terrestris* XP_003397025.1, *Apis mellifera* BAC76400.1, *A. florea* XP_012344213.1, *Habropoda laboriosa* KOC70401.1, *Acromyrmex echinaior* EGI65606.1, *Fopius arisanus* XP_011313683.1, *Bombyx mori* NP_001124364.1, *Chilo suppressalis* ALM30345.1, *Drosophila melanogaster* NP_650141.2, *Cimex lectularius* XP_014240732.1, *Tribolium castaneum* KYB25859.1, *Locusta migratoria* AKN21238.1, *Zootermopsis nevadensis* KDR18855.1, *Periplaneta americana* AAX11212.1, *Blattella germanica* CUT08829.1, *Rhyarobia maderae* AAX11211.2) using the online tool MUSCLE 3.8 (Multiple Sequence Comparison by Log-Expectation www.ebi.ac.uk/Tools/msa/muscle). A phylogenetic tree was generated using the Maximum Likelihood method, based on the Whelan and Goldman model [47], on the Molecular Evolutionary Genetics Analysis 6 software (MEGA6) [44]. The tree is presented along with bootstrap values based on 1000 replicates.

2.5. Transcript expression analysis by quantitative PCR

Unfed 5th instars were dissected under physiological saline prepared using nuclease-free water and the tissues were pooled in 7 groups: CNS, salivary glands (SG), fat body (FB), dorsal vessel (DV), foregut (FG), midgut (MG) and hindgut (HG). Total RNA was extracted using a PureLink RNA mini kit (Life Technologies Corporation, Carlsbad, CA, USA), and quantified using a NanoDrop System (ND-1000 Thermo Scientific, Burlington, ON, Canada). Using 100 ng of total RNA, the cDNA was synthesized from each tissue using iScript Reverse Transcription Supermix (Bio-Rad, Mississauga, ON, Canada). The cDNA was subsequently diluted with nuclease free water and 5 ng were used per well as template for quantitative PCR (qPCR) reactions, which were carried out on a Mx3005P Quantitative PCR System (Stratagene, Mississauga, ON, Canada) using SsoFast EvaGreen Supermix with low ROX (Bio-Rad, Mississauga, ON) following manufacturer suggested cycling conditions. The Rhopr-TK cDNA fragment was amplified using the forward primer 5' TTCCGGCTTCATGGGCGTCA 3', and the reverse primer 5' CCTGTTTAAGAGCTGATTCACG 3'. Primers were optimized to amplify target fragments of similar size in Rhopr-TK as well as in the reference genes (*rp49*, *β-actin* and *α-tubulin*) [26]. The dissociation of the reaction products rendered a single peak for each primer set in the

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