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Isolation and characterization of the corticotropin-releasing factor-related diuretic hormone receptor in *Rhodnius prolixus*

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ABSTRACT

Rhodnius prolixus, the vector of human Chagas disease, is a hemipteran insect that undergoes rapid post-feeding diuresis following ingestion of a blood meal that can be up to 10 times its initial body weight. Corticotropin-releasing factor-related diuretic hormone (Rhopr-CRF/DH) and serotonin are neurohormones that are synergistic in increasing rates of fluid secretion by Malpighian tubules during this rapid post-feeding diuresis. A Rhopr-CRF/DH receptor transcript has now been isolated and characterized from fifth instar *R. prolixus*. The receptor is a family B1 (secretin) G protein-coupled receptor (GPCR) and was deorphaned in a heterologous cellular system using Chinese hamster ovary (CHO) cells stably expressing a promiscuous G-protein (Gα16). This assay was also used to demonstrate the presence of Rhopr-CRF/DH in the haemolymph of *R. prolixus* in response to blood-gorging. Two additional cell lines were used in this heterologous assay to verify that the cyclic adenosine monophosphate (cAMP) pathway and not the inositol triphosphate (IP_3) pathway was stimulated upon activation of the receptor. Lastly, quantitative PCR demonstrated strong receptor expression in digestive tissues, upper Malpighian tubules and reproductive tissues. Identification of the Rhopr-CRF/DH receptor now provides tools for a more detailed understanding into the precise coordination of diuresis and other physiological processes in *R. prolixus*.

1. Introduction

When the blood-feeding bugs, *Rhodnius prolixus*, gorge on a blood meal, they initiate rapid and prolonged diuresis to reduce the bulk of their mass and to concentrate the nutrients of the blood meal, while maintaining volume, osmotic and ionic balance of the hemolymph [1–5]. During this diuresis, *R. prolixus* acts as the vector of human Chagas disease by transmitting the parasite *Trypanosoma cruzi* in the urine, close to the feeding site. Rapid diuresis begins within 2–3 min of feeding and lasts for the next 3 h at a high rate of 400–700 nL/min, during which time over 50% of the mass of the blood meal is excreted as urine [3–5]. Water, sodium ions and chloride ions from the ingested blood are absorbed into the hemolymph across the anterior midgut (AMG). This is countered by the upper Malpighian tubules (MTs), which secrete sodium, potassium, and chloride ions and water, resulting in an isoosmotic fluid that is rich in potassium ions. As the fluid moves down to the lower MTs, potassium and chloride ions are reabsorbed with minimal water into the hemolymph and hypo-osmotic primary urine that is low in potassium and high in sodium empties into the hindgut for elimination [6–8]. This entire process in *R. prolixus* is largely controlled by diuretic hormones released from neurosecretory cells (NSCs) found within the central nervous system (CNS) [2–4,8].

Insect diuretic hormones include the biogenic amines, tyramine and serotonin, and multiple families of neuropeptides, such as corticotropin-releasing factor (CRF)-related diuretic hormone (CRF/DH), calcitonin (CT)-like diuretic hormone (CT/DH), kinins and cardioacceleratory peptide 2b (CAP_{2b}) [9]. Only two insect diuretic hormones have been shown to be released into the hemolymph in response to feeding. These are serotonin in *R. prolixus* [10] and the CRF/DH in *Locusta migratoria* [11]; however, many of the other neuropeptides possess diuretic activity.

Corticotropin-releasing factor/hormone is commonly known as the stress hormone in vertebrates but CRF orthologs act as diuretic peptides in insects [12]. The first insect CRF/DH was sequenced from *Manduca sexta* where diuretic effects were shown *in vivo* [13]. Since then, other CRF/DHs have been sequenced from *Periplaneta americana* [14],

Abbreviations: AMG, anterior midgut; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin; CT, calcitonin; CAP2b, cardioacceleratory peptide 2b; CNS, central nervous system; CHO, Chinese hamster ovary; CRF, corticotropin-releasing factor; cAMP, cyclic adenosine monophosphate; CNG, cyclic nucleotide-gated; DH, diuretic hormone; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; G□16, G protein alpha 16; IP3, inositol trisphosphate; MT, Malpighian tubules; MTGM, mesothoracic ganglionic mass; NSCs, neurosecretory cells; PDF, pigment-dispersing factor; PKA, protein kinase A; PLC, phospholipase C; qPCR, Quantitative Polymerase Chair Reaction; TEP, transepithelial potential; TMB-8, 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride.

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L. migratoria [15], *Drosophila melanogaster* [16], *R. prolixus* [17]and a second CRF/DH from *M. sexta* [18]. CRF/DHs have been shown to increase fluid excretion *in vivo* and *in vitro* and to increase cAMP content in MTs [14,15,19,20]. Besides diuretic effects, CRF/DH has been shown to induce satiety in *M. sexta* and *Schistocerca gregaria* [21,22], initiate pre-ecdysis behavior in *M. sextra* [23] retard reproduction in *Schistocerca gregaria* [22], modulate normal rest: activity rhythms, as well as control sperm ejection and storage in *D. melanogaster* [24,25].

The CRF/DH in *R. prolixus* (Rhopr-CRF/DH) is 49 amino acids long and is found in NSCs in the brain and in the mesothoracic ganglionic mass (MTGM) as well as their associated neurohemal sites [17,26]. Physiologically, Rhopr-CRF/DH increases secretion rates in MTs and increases the rate of absorption across the AMG [17]. Rhopr-CRF/DH acts synergistically with serotonin to increase secretion rates *via* the cAMP second messenger pathway [27–29]. Unlike serotonin, Rhopr-CRF/DH has no effect on the reuptake of potassium ions in the lower MTs [30]. Rhopr-CRF/DH also stimulates contractions of *R. prolixus* hindgut [31].

CRF/DH receptors belong to the family of secretin-like GPCRs and are categorized under subfamily B1 that primarily use cAMP as their secondary messenger. Other members of this family include CT/DH and pigment-dispersing factor (PDF) receptors in insects [32]. Parallel to its ligand, CRF/DH receptors have been isolated and cloned in a number of insect species. Like the CRF/DH peptide, the *M. sexta* CRF/DH receptor was the first to be cloned, closely followed by the sequencing of the CRF/DH receptor in *Acheta domesticus* [33,34]. Other insect CRF/DH receptor orthologs have been found including two *Drosophila* CRF/DH (also known as DH_{44}) receptors: DH_{44}-R1 (encoded by CG8422) and DH_{44} – R2 (encoded by CG12370) [35,36]. The first *Drosophila* CRF/DH receptor (DH_{44}-R1) stimulates increases in both cAMP and calcium ions upon receptor activation, while the second (DH_{44}-R2) only increases levels of cAMP [35,36].

Here we have isolated and characterized the Rhopr-CRF/DH receptor transcript from fifth instar *R. prolixus*. The receptor was deorphaned using a heterologous cellular assay. This assay was also used to demonstrate the presence of Rhopr-CRF/DH in the hemolymph of *R. prolixus* in response to blood-gorging. Quantitative PCR (qPCR) demonstrates strong receptor expression in digestive and reproductive tissues. We have also verified that this receptor couples to the cAMP second messenger pathway.

2. Materials and methods

2.1. Animals

Fifth instar and adult *R. prolixus* were maintained in incubators at 25 °C and 60% humidity. Insects were routinely fed through a membrane on defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA, USA; supplied by Cedarlane Laboratories Inc., Burlington, ON, Canada) once during each instar. Tissues were dissected from insects 4–6 weeks post-feeding as the previous instar, under nuclease-free phosphate-buffered saline (PBS) (Sigma-Aldrich, Oakville, ON, Canada).

2.2. Molecular cloning

D. melanogaster CRF/DH receptor protein sequences (CG12370, isoform A accession no. NP_725175.3 and isoform B accession no. NP_610789.3) were used as the query to search the *R. prolixus* preliminary genome assembly (June 2009 release). A forward gene-specific primer (CRFR2 FOR1: 5′ GGTCTACTCTGTTGGCCGAATAC 3′) and a reverse gene-specific primer (CRFR2 REV 1: 5′ AGATAAGCGAG CAAGGTTTGTACTAC 3′) were designed based on the resultant hits and used to amplify the partial cDNA sequences encoding Rhopr-CRF/DH-R2A and B using a fifth instar *R. prolixus* CNS cDNA library [37] as the template. PCR was performed using an S1000 thermal cycler (Bio-Rad Laboratories, Mississauga, ON, Canada) with the following temperature cycling profile: initial denaturation (94 °C for 3 min) then 40 cycles of denaturation (94 °C for 30 s), annealing (57 °C for 30 s) and extension (72 °C for 2 min) and a final extension for 10 min at 72 °C. Positive amplicons were cloned and sent for sequencing at the Centre for Applied Genomics at the Hospital for Sick Children (MaRS Centre, Toronto, ON, Canada) as previously described [38]. Since Rhopr-CRF/DH-R2A encoded a protein that comprised only six transmembrane domains (data not shown; partial sequence submitted to NCBI under the accession number: KU942308) and was thus atypical, we focused on Rhopr-CRF/DH-R2B from hereon. The 5′ and 3′ untranslated regions of Rhopr-CRF/DH-R2B cDNA were obtained using a modified 5′ and 3′ rapid amplification of cDNA ends (RACE) PCR technique as described [38]. Rhopr-CRF/DH-R2B cDNA specific 5′ and 3′ RACE primers are listed in Tables 1 and 2 respectively. The full sequence was amplified using primers listed in Table 3, cloned and sent for sequencing as described above. The sequence has been submitted to NCBI under the accession number: KJ407397.

Table 1
5′ RACE primers.

Primer	Sequence(5′-3′)
CRFR2raceRO	TTTGGTTTGAAGTAAGAGTGTGTAG
CRFR2raceR1	GCCCAAGTACCATTTTCATAGC
CRFR2raceR2	ACACAGGGTAGATAAGCCGTG
CRFR2raceR3	GGTGTATTCGGCCAACAGAG

Table 2
3′ RACE primers.

Primer	Sequence (5′-3′)
CRFR2raceF1	CTGATGGTAGCGTTGTTATACTGC
CRFR2raceF2	GTAACCTGGGAGCAAGACG
CRFR2raceF3	GTCTCCAAATACCAGAACTGAAAGTG
CRFR2raceF4	CAGTGGCCAGCGAGAATAC

2.3. Sequence analysis

Rhopr-CRF/DH-R2 gene structure was determined using WebScipio [39]. Membrane topology of Rhopr-CRF/DH-R2A and B protein sequences was predicted using the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The potential phosphorylation sites were predicted using NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos/) and potential N-linked glycosylation sites were predicted using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) was used to align Rhopr-CRF/DH-R2A and B protein sequences with the following CRF and CRF/DH receptors: M. sexta (AAC46469.1), *A. domesticus* (AAC47000.1), *D. melanogaster* (NP_610960.1 and NP_610789.3) and *Homo sapiens* (NP_001138618.1 and NP_001874.2). The alignment figure was obtained from BOXSHADE 3.21 server (http://www.ch.embnet.org/software/BOX_form.html). The secondary structure was predicted using Phyre V. 2. 0 (www.sbg.bio.ic.ac.uk/phyre2/) and analyzed using the UCSF Chimera software (http://www.cgl.ucsf.edu/chimera).

Phylogenetic analysis of deuterostomian CRF and protostomian CRF/DH receptors was performed using maximum-likelihood. Briefly, sequences were aligned using a MAFFT v7.017 plugin in Geneious 8.0.5 [40] and the alignment was trimmed using BMGE [41]. Maximum-

Table 3
Primers to amplify the complete sequence.

Primer	Sequence (5′-3′)
FOR-CRFR2B	CATCTTGAATACATTCGTGACG
REV-CRFR2B	CAAAAATTCTACTTCACCACTTAGAC