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Molecular and functional characterization of the first tick CAP_{2b} (periviscerokinin) receptor from *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae)

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ABSTRACT

The cDNA of the receptor for CAP_{2b}/periviscerokinin (PVK) neuropeptides, designated *Rhimi-CAP_{2b}-R*, was cloned from synganglia of tick *Rhipicephalus* (*Boophilus*) *microplus*. This receptor is the ortholog of the insect CAP_{2b}/PVK receptor, as concluded from analyses of the predicted protein sequence, phylogenetics and functional expression. Expression analyses of synganglion, salivary gland, Malpighian tubule, and ovary revealed *Rhimi-CAP_{2b}-R* transcripts. The expression in mammalian cells of the open reading frame of *Rhimi-CAP_{2b}-R* cDNA fused with a hemagglutinin tag at the receptor N-terminus was confirmed by immunocytochemistry. In a calcium bioluminescence assay the recombinant receptor was activated by the tick *Ixodes scapularis* CAP_{2b}/PVK and a PVK analog with EC_{50s} of 64 nM and 249 nM, respectively. Tick pyrokinins were not active. This is the first report on the functional characterization of the CAP_{2b}/PVK receptor from any tick species which will now permit the discovery of the physiological roles of these neuropeptides in ticks, as neurohormones, neuromodulators and/or neurotransmitters.

1. Introduction

Being obligate blood feeders, ticks are vectors of pathogens to humans, animals and livestock (Anderson and Magnarelli, 2008; Sonenshine, 1993). The southern cattle tick, *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) is the world's most economically important tick which transmits the haemoparasites that cause cattle fever and anaplasmosis (Angus, 1996). The control of ticks depends heavily on pesticides targeting the nervous system or inhibiting chitin deposition in the cuticle (Lees and Bowman, 2007). Multiple resistance to these acaricides has become widespread (Guerrero et al., 2012; Pérez de León et al., 2012), thus, research on validating new molecular targets, including those in the endocrine system that could serve for developing new selective acaricides is needed (Holmes et al., 2003; Lees and Bowman, 2007).

Neuropeptides and their receptors play a critical integrative role in the regulation of virtually all physiological processes in insects such as reproduction, growth, feeding, locomotion, and excretion (Caers et al., 2012). Insect researchers have made an appreciable progress in finding novel neuropeptides and their receptors, and understanding their physiological functions for advancing invertebrate endocrinology (Caers et al., 2012; Kwon et al., 2012). The first cloning of a tick neuropeptide receptor for invertebrate kinin(s) (i.e. myokinin) was reported in 2000 and its functional analysis reported in 2003 (Holmes et al., 2003; Holmes et al., 2000). Recently the characterization of myoinhibitory peptide and SIFamide receptors has been released (Šimo et al., 2013). The functional analyses of tick biogenic amine receptors have been published previously (Baxter and Barker, 1999; Chen et al., 2004; Chen et al., 2007; Meyer et al., 2011). Transcriptomics and *in silico* analyses predictions of hard tick neuropeptides and their receptors are providing candidates for physiological studies (Bissinger et al., 2011; Christie, 2008; Donohue et al., 2010; Sonenshine et al., 2011). Further, the immunolocalization of tick neuropeptides is being accomplished with antibodies generated against insect neuropeptides (Šimo et al., 2009a). Despite this progress, only one report is available on tick neuropeptidomics (Neupert et al., 2009) while attempts at tick neuropeptide peptide isolation are rare (Liang et al., 2005).

Ticks, being pool blood feeders that feed continuously after attachment cope with a variable intake of blood, minimal to moderate during the early phases of feeding to large during the late feeding phase. However, as the meal is digested, they must simultaneously maintain fluid and ion homeostasis. Similar to haematophagous insects, this complex regulation is likely achieved via

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a number of neuropeptides and their receptors (Holmes et al., 2003; Holmes et al., 2000; Šimo et al., 2013; Šimo et al., 2009b). We began investigating the cardioacceleratory peptide 2b (CAP_{2b})/periviscerokinin (PVK) system to reveal its physiological roles in ticks because of its proven involvement in the regulation of fluid excretion in insects (Neupert et al., 2005). The first CAP_{2b}/PVK peptide identified in insects was isolated from the moth *Manduca sexta* and exhibited cardioactive activity for which it was named Manse-CAP_{2b}, CAP indicating cardioacceleratory peptide (Huesmann et al., 1995; Wegener et al., 2002). Later, Davies et al. (1995) found that *M. sexta* CAP_{2b} could stimulate fluid secretion from Malpighian tubules, the renal organs of the fruit fly *Drosophila melanogaster*; the diuretic effect of this peptide on Malpighian tubules later was confirmed in various insect species (Nachman et al., 2006; Pollock et al., 2004; Predel et al., 2006). Conversely, PVKs act as antidiuretic hormones in the kissing bug *Rhodnius prolixus* and other insect species (Coast and Garside, 2005; Paluzzi and Orchard, 2006). Recently it was found that in larvae of *Aedes aegypti* mosquitoes, PVK peptide could induce either diuretic or antidiuretic activity in isolated Malpighian tubules depending if applied at high or low concentration, and acting through the NOS/cGMP/PKG or PKA pathways, respectively (Ionescu and Donini, 2012).

Previous to the sequencing of the tick *Ixodes scapularis* genome, a first attempt to identify the tick CAP_{2b}/PVK peptides was with two tick species *Ixodes ricinus* and *R. microplus* (Neupert et al., 2005), pioneering the application of MALDI-TOF/TOF and tandem mass spectrometry. This effort resulted in the identification of a partially correct sequence (underlined) within the reported PALIPFPRVa. Later, a successful identification of the CAP_{2b}/PVK peptide sequence from *I. scapularis* was verified by Neupert et al. (2009) as pQGLIPFPRVa (Christie, 2008). Yet, the cognate receptor has not been identified to begin to decipher the unknown physiological roles of CAP_{2b}/PVK peptide in ticks.

Receptors for insect CAPA-related peptides have been characterized in insects (Iversen et al., 2002; Olsen et al., 2007; Paluzzi et al., 2010; Park et al., 2002). The insect CAP_{2b}/PVK receptor which belongs to PRXamide peptide receptor family is a G protein-coupled receptor (GPCR) evolutionarily related to human neuromedin U receptor (Iversen et al., 2002; Park et al., 2002; Terhzaz et al., 2012). Knowledge of the function of CAP_{2b}/PVKs in ticks will be advanced by unequivocally identifying its receptor. However, the identity of the tick CAP_{2b}/PVK receptor is still unknown. We have cloned and functionally characterized the first tick CAP_{2b}/PVK receptor from the synganglion of *R. microplus*. We have also investigated the tissue transcriptional expression profile in females partially fed for 5 days.

2. Materials and methods

2.1. Ticks and tissues

The pesticide-susceptible Gonzalez strain of the southern cattle tick *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini) was used for these experiments (Holmes et al., 2000). This strain was obtained from the Cattle Fever Tick Research Laboratory, USDA-ARS, Mission, TX, USA under a cooperative agreement with Texas A&M AgriLife Research. Bovines were infested with 250 mg of larvae confined in patches symmetrically placed on both sides of the host and allowed to molt to adults as described (Brake et al., 2010). Females partially fed for five days were collected after 19 days of the initial larval infestation. For cloning and transcriptional analyses, females were dissected under physiological saline to obtain tissues including midgut, Malpighian tubules, salivary gland, synganglion, ovary and rectal sac, and tissues were immediately placed in RNAlater® solution (Ambion®, Life Technologies, Calsbad, CA, USA) at 4 °C until processed for mRNA extraction within 2–3 days.

2.2. RT-PCR and RACE PCRs

To clone the cDNA corresponding to the central region of the open reading frame (ORF) of *R. microplus* CAP_{2b}/PVK receptor (*Rhimi-CAP_{2b}-R*), a specific forward primer P21 (see Table S1 for primers sequences) was designed based on the partial sequence of putative *I. scapularis* CAP_{2b}/PVK receptor (*IxoscGPRcap*; VectorBase No.: ISCW012018). A degenerate reverse primer (P25) was designed from the conserved regions of another partial sequence of candidate *IxoscGPRcap* (VectorBase No.: ISCW014181) and insect CAP_{2b}/PVK receptors (Jurenka and Nusawardani, 2011). First, whole tick mRNA was isolated from partially fed females using the DynaBeads® mRNA direct kit (Invitrogen™, Life Technologies, Carlsbad, CA, USA); 0.5 μg mRNA was used for cDNA synthesis (21 μl final volume) using SuperScript® III First-Strand Synthesis System (Invitrogen) following the manufacturer's specification. This cDNA was used as template to amplify the initial *Rhimi-CAP_{2b}-R* receptor fragment (558 bp in length) with primers P21 and P25. The PCR reaction (50 μl final volume) contained 2 μl cDNA, 1 μl Advantage® 2 Polymerase Mix (Clontech, Mountain View, CA, USA), 0.4 μM of each primer, 0.2 mM dNTPs, 1X Advantage® 2 PCR buffer. PCR conditions were as follows: initial denaturation and enzyme activation at 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 65.6 °C for 30 s, and 72 °C for 1 min then a final extension of 72 °C for 5 min. PCR products were purified using QIAquick Gel Extraction kit (Qiagen, Valencia, CA, USA), and cloned into pCR™2.1 Vector (Invitrogen). Competent cells (One Shot® Top 10F', Invitrogen) were transformed with the cloned PCR products and grown; plasmids were isolated by QIAprep Spin Miniprep kit (Qiagen) and sequenced by the Gene Technology Laboratory (Texas A&M University, College Station, TX, USA). All cDNA clones were sequenced at least twice.

Preliminary RT-PCR analyses revealed clear expression of a single band in synganglia (data not shown). Therefore, to obtain the full length cDNA from synganglia, 11 ticks were dissected and mRNA was isolated with DynaBeads® mRNA direct kit as indicated above. For 5' and 3' RACE PCRs, RACE-ready cDNAs were synthesized using SMARTer™ RACE cDNA Amplification kit (Clontech) (110 μl final volume). Four specific primers (Table S1) were designed to amplify the 5' and 3' ends of the *Rhimi-CAP_{2b}-R* cDNA based on the sequence of the cloned ORF central region. All PCR reactions were carried out in 50 μl volumes. For 5' or 3' RACE, primary amplification was as follows: 2 μl of 5' or 3' RACE-ready cDNA of synganglia was added into the mix of 1X Advantage® 2 buffer, 0.2 mM each dNTPs, 0.4 μM of UPM (Clontech) and P28 (for 5' RACE) or Rm57sF (for 3' RACE), and 1 μl Advantage® 2 Taq DNA polymerase (Clontech). The nested amplification was as follows: to reduce non-specific products, 2 μl of primary amplification products were diluted in 98 μl of water, of which 2 μl was added into the mix of 1X Advantage® 2 buffer, 0.2 mM each dNTPs, 0.4 μM NUP and P29 (for 5' RACE) or Rm59sF (for 3' RACE), and 1 μl Advantage® 2 Taq DNA polymerase. For both 5' and 3' RACE, PCR conditions were as follows: 5 cycles (94 °C for 30 s, 72 °C for 3 min), followed by 5 cycles (94 °C for 30 s, 70 °C for 30 s, 72 °C for 3 min), followed by 40 cycles (94 °C for 30 s, 68 °C for 30 s, 72 °C for 3 min) with a final extension step of 72 °C for 5 min. The resulting bands were purified, cloned and sequenced as described above.

2.3. Sequence analyses

Lasergene software (DNASTAR, Madison, WI, USA) was used for analyzing sequences obtained from the sequencing center. The