FEBS Letters 585 (2011) 3507–3512

journal homepage: www.FEBSLetters.org

Mosquito *Aedes aegypti* (L.) leucokinin receptor is critical for *in vivo* fluid excretion post blood feeding

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ARTICLE INFO

Article history:
Received 20 July 2011
Revised 7 September 2011
Accepted 4 October 2011
Available online 15 October 2011

Edited by Gianni Cesareni

Keywords:
Insect GPCR (G protein-coupled receptor)
Diuresis
Malpighian tubules
RNAi
Rectum
Midgut

ABSTRACT

The evolution of the blood feeding adaptation in mosquitoes required hormonal coordination of multiple physiological processes (behavior, digestion, diuresis, oogenesis). The *Aedes* kinins (leucokinin-like neuropeptides) are involved in post blood feeding physiological processes, having diuretic and myotropic functions. To understand the *in vivo* contribution of the kinin receptor to overall female post-prandial fluid excretion, RNAi knockdown was followed by fluid secretion assays which proved its fundamental role in rapid diuresis. The *Aedes* kinin receptor was also localized in several tissues not previously reported in mosquitoes. Results highlight the integrative role of the *Aedes* kinins in the success of the blood feeding adaptation.

1. Introduction

In mosquitoes, the evolution of the blood feeding adaptation required precisely coordinating multiple physiological processes including behavior, digestion, excretion and oogenesis [1]. While protein from the blood meal is required for reproductive success, females ingest more than ten times their hemolymph volume in blood, imposing an osmoregulatory challenge and likely a fitness cost due to potential predation due to the added weight hindering immediate flight. Accordingly, females retain essential proteins in the midgut while excess ions and fluid are removed from the hemolymph and excreted from the body by the Malpighian tubules and hindgut. The rectum section of the hindgut has osmoregulatory pear-shaped ultrastructures called rectal papillae. These anatomically and functionally related tissues are under synchronous hormonal control [2]. It is therefore plausible that known diuretic hormones may have simultaneous actions on other organs, rendering them integrative signaling molecules.

While numerous hormones function after a blood meal, leucokinin-like neuropeptides are of particular interest because of their multifunctional diuretic and myotropic activities and their potential role in digestive enzyme release in other insects [3–5]. In *Aedes aegypti* three leucokinin-like peptides have been identified, *Aedes* kinins I, II, and III, which are released from the abdominal ganglia, interact with a single *Aedes* kinin receptor (*Ae*KR) and stimulate both hindgut contractions and chloride and fluid flux toward the Malpighian tubule lumen for primary urine production [4,6–9]. The *Ae*KR (ID AAT95982.1) is predicted as a 65.2 kDa G protein-coupled receptor (GPCR) that signals through the intracellular Ca^{2+} cascade [9,10]. To date, work to elucidate the contribution of the *Ae*KR to physiological processes in *A. aegypti* has focused on the renal organs, the Malpighian tubules, where the stellate cells express the *Ae*KR [11]. In other arthropods, leucokinin-like peptides and/or their receptor have been discovered in the gonads, midgut and nervous system [3,12,13].

This study aimed to assess the receptor's tissue distribution and evaluate its role in post blood feeding diuresis. This is the first comprehensive *Ae*KR analysis in mosquito tissues and the first report demonstrating its *in vivo* contribution to post-prandial fluid excretion.

2. Materials and methods

2.1. Insects and tissues

A. aegypti (L.) (Diptera: Culicidae) Rockefeller strain was maintained as described [11]. Females were blood fed using water-jacketed feeders (Chemglass Life Sciences, NJ, USA) [14] with defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA, USA).

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0014-5793/$36.00
doi:10.1016/j.febslet.2011.10.001

2.2. Western blot

Tissue membrane preparations from 500–1000 females and Western blots were performed as described [11]. Membranes were prepared from the head, posterior midgut, M. tubules, hindgut, and ovaries of non-blood fed (NBF) and blood fed (BF) females 3–5 days post eclosion (PE). Dissections of BF females were completed 1–3 h post feeding. Ovaries were also dissected 24 h after feeding. For NBF tissues 100 μg of membrane preparations were loaded per lane and for BF females, 50 μg/lane were used. Transferred proteins were probed with either anti-KR-$Ct_{328-345}$ antibodies (1:250) or anti-KR-$Ct_{328-345}$ antibodies (1:250) pre-absorbed overnight with 500 μg C-terminal peptide antigen, as negative controls.

2.3. Immunohistochemistry (IHC)

Whole-mount IHC was completed and analyzed by fluorescent and confocal microscopy as described [11] with the anti-KR-$Ct_{328-345}$ antibody (1:10) or the anti-KR-$Ct_{328-345}$ antibody pre-absorbed with 500 μg of the C-terminal peptide antigen (1:10) for the posterior midgut, rectum, and M. tubules from NBF females 3–5 days PE. IHC of wax sections (12 μm) was performed as described [15]. Posterior midgut sections were incubated with the anti-KR-$Ct_{328-345}$ antibody (1:40) or the anti-KR-$Ct_{328-345}$ antibody pre-absorbed with 500 μg of the C-terminal peptide antigen (1:40) at 4 °C overnight.

2.4. dsRNA synthesis and injections

Template DNA for dsRNA synthesis was prepared using an *A. aegypti* KR cDNA clone (AY596453) with primers Aa-KRdsRNA-T7F (5′-TAATACGACTCACTATAGGGACCGAAGTGGATTTCAAGTGGTTGGAGGTG-3′) and Aa-KRdsRNA-T7R (5′-TAATACGACTCACTATAGGGCATCGCTGCCGTTCAGTGTATTGTTGTTTGC-3′) with T7 regions (underlined), and amplified by PCR (nucleotides 354–901). MEGAscript®RNAi Kit (Ambion, Austin, TX, USA) was used for dsRNA synthesis then de-salted and concentrated using a Microcon column (Milipore, Billerica, MA, USA). All dsRNA was diluted in nuclease-free water to 8 μg/μl. DsRNA (2 μg) was injected into the thorax of cold anesthetized females 24 h PE using an Eppendorf FemtoJet® Micro-Injector (Eppendorf, Hauppauge, NY, USA) equipped with an Eppendorf TransferMan NK Micromanipulator. Mosquitoes were allowed to recover overnight before males were added for mating.

2.5. Quantitative PCR

Females (n = 50–60) were dissected five days post injection and M. tubules, hindguts, and posterior midguts were stored in RNAlater Solution (Ambion). Tissues were transferred to TRizol (Invitrogen, Carlsbad, CA, USA) and total RNA was extracted per manufacturer's protocol. After RNA was treated with DNase I (Invitrogen) the TRIzol protocol was repeated. Total RNA was solubilized in 15 μl nuclease-free water with 1 μl RNaseOUT (Invitrogen). cDNA was synthesized using the SuperScript™ III First-Strand Synthesis System (Invitrogen) using ~3 μg total RNA as template and random hexamer primers for a final volume of 20 μl per cDNA reaction. The amplicon (*Ae*KR bp 1340–1398) was amplified with primers AaKR_qPCR_F (5′-TGGCCGCTCCAACTCTGT-3′) and *Aa*KR_qPCR_R (5′-TGTCAATCGTTAGCCTGGGC-3′). BLAST search of this region resolved no similarity to other regions of the *A. aegypti* genome. The 18S rRNA gene was amplified using primers 18S_rRNA_(forward) and _rRNA_(reverse) [16] for normalization. Primer and cDNA concentrations were optimized. Quantitative PCR was performed by an ABI-7300 (Applied Biosystems, Carlsbad, CA, USA) using POWER/SYBR® Green Master Mix (Applied Biosystems). The comparative threshold cycle (C_T) method was used to assess transcript levels. Results of four independent experiments were analyzed by t-test in PASW Statistics 18 (SPSS Inc., Somers, NY, USA) to a 95% confidence interval (CI).

2.6. AeKR RNA interference (RNAi) evaluation by in vivo fluid excretion assay

Injected females, 5–9 days post-treatment, were blood fed one-at-a-time then placed into a precision humidity chamber with constant flow of dry air (100 ml/min) to measure excretion [17]. Humidity readings for the chamber were recorded every 0.01 s for 60 min by an RH-300 flow-through humidity analyzer (Sable Systems, Henderson, NV, USA) at RT. System calibration was performed by applying known volumes of water (0.5–1.5 μl) to the chamber. Results were analyzed in Expedata (Sable Systems) followed by Repeat Measures ANOVA (95% CI).

3. Results and discussion

3.1. Tissue-specific expression of the AeKR

A 75 kDa band confirmed the presence of the *Ae*KR in the M. tubules (MT), head, posterior (post.) midgut, hindgut, and ovaries of NBF and BF females (Fig. 1). This band, 10 kDa higher than the predicted 65 kDa protein, was previously recognized in the M. tubules where the size difference was attributed to post-translational modifications [9,11]. Treatment with PNGaseF to test for receptor N-linked glycosylation revealed no shift in band size (not shown). However, other post-translational modifications may explain the observed size disparity as Pietrantonio et al. [9] noted three potential ASNglycosylation sites, 34 prospective phosphorylation sites and five possible palmitoylation sites in the protein sequence. A weaker band around 40 kDa, which has previously been considered a degradation product, was also resolved in all samples except the NBF and 1–3 h PBF ovaries. It appears this band is stronger in protein preparations from tissues 1–3 h post blood feeding, which may suggest more rapid degradation after a blood meal and that the receptor population may be regulated after feeding. The presence of other receptor isoforms was ruled out by previous extensive cloning and sequencing [11]. Additional bands were observed in the ovaries (NBF and BF). A prominent doublet at 150 kDa suggests the receptor may dimerize in the ovaries. Two further bands were observed just below and above the 75 kDa band. As posttranslational modification of GPCRs is known to regulate their activity [18], alternate modification of the *Ae*KR in the

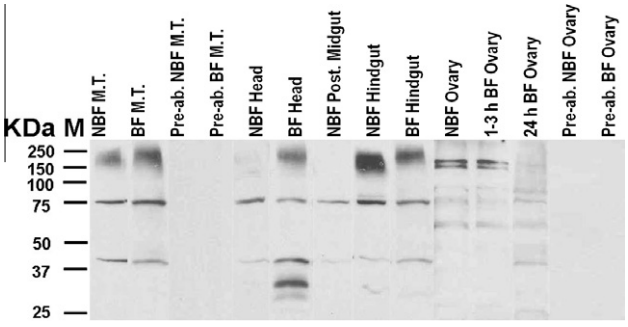

Fig. 1. Western blot analysis of the *Ae*KR. The receptor was detected as a 75 kDa protein band in all membrane preparations with an anti-KR-$Ct_{328-345}$ antibody. Anti-KR-$Ct_{328-345}$ antibody preabsorbed with the C-terminal peptide antigen (Pre-ab.) served as a negative control. Additional bands at ~15–200 kDa in the ovary could represent receptor aggregation, dimerization, or binding to other unknown proteins. 100 and 50 μg of membrane preparations were loaded per lane from tissues of non-blood-fed and post blood-feed females, respectively.