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Ecdysis triggering hormone signaling in the yellow fever mosquito *Aedes aegypti* ☆

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ABSTRACT

At the end of each developmental stage, the yellow fever mosquito *Aedes aegypti* performs the ecdysis behavioral sequence, a precisely timed series of behaviors that culminates in shedding of the old exoskeleton. Here we describe ecdysis triggering hormone-immunoreactive Inka cells located at branch points of major tracheal trunks and loss of staining coincident with ecdysis. Peptides (AeaETH1, AeaETH2) purified from extracts of pharate 4th instar larvae have—PRXamide C-terminal amino acid sequence motifs similar to ETHs previously identified in moths and flies. Injection of synthetic AeaETHs induced premature ecdysis behavior in pharate larvae, pupae and adults. Two functionally distinct subtypes of ETH receptors (AeaETHR-A, AeaETHR-B) of *A. aegypti* are identified and show high sensitivity and selectivity to ETHs. Increased ETHR transcript levels and behavioral sensitivity to AeaETHs arising in the hours preceding the 4th instar larva-to-pupa ecdysis are correlated with rising ecdysteroid levels, suggesting steroid regulation of receptor gene expression. Our description of natural and ETH-induced ecdysis in *A. aegypti* should facilitate future approaches directed toward hormone-based interference strategies for control of mosquitoes as human disease vectors.

1. Introduction

During growth and development, all insects undergo ecdysis, the periodic shedding of the exoskeleton to increase their body size and facilitate morphological changes during metamorphosis. This intricate, yet life-threatening process requires precisely-timed developmental scheduling under the control of steroid and peptide hormones. To execute the sequence successfully, a peptide signaling cascade is programmed through steroid-induced gene expression to schedule an innate, stereotypic behavior. Recent reviews summarize our understanding of this process in moths and flies (Truman, 2005; Zitnan and Adams, 2005; Zitnan et al., 2007).

Elevation of 20-hydroxyecdysone (20E) at the end of the feeding phase of each instar leads to apolysis and initiation of the molt, during which the old cuticle is broken down and recycled into new cuticle. Subsequent decline of 20E is essential for initiation of the ecdysis sequence, which terminates the molt (Kingan and Adams, 2000; Truman et al., 1983; Zitnan et al., 1999). In *Manduca sexta*, release of ecdysis triggering hormones (PETH and ETH) from endocrine Inka cells initiates pre-ecdysis and ecdysis behaviors (Zitnan et al., 1999). Two ETH homologs, DrmETH1 and DrmETH2, were subsequently identified in *Drosophila melanogaster* (Park et al., 1999). ETH null mutants showed lethal ecdysis defects, indicating that ETH peptides are both necessary and sufficient to initiate the ecdysis behavioral sequence (Park et al., 2002). ETH peptides act directly on the central nervous system (CNS) to trigger a neuropeptide signaling cascade, which includes eclosion hormone (EH), kinins, diuretic hormones, crustacean cardiactive peptide (CCAP), myoinhibitory peptides (MIP), and bursicon. These peptides together with ETH recruit central pattern generators that drive pre-ecdysis, ecdysis, and post-ecdysis behaviors (Kim et al., 2006a,b). The production and release of ETH and PETH in Inka cells are regulated by 20E levels (Zitnan et al., 1999; Zitnanova et al., 2001). In addition, central neuronal circuits are also regulated by 20E. For example, competence of the CNS to respond to ETH is under steroid control (Zitnan et al., 1999; Zitnanova et al., 2001). Further investigation of the role ecdysteroids play in CNS sensitivity to ETHs is needed.

Inka cells and ETH homologs are widely distributed in all major insect orders (Zitnan et al., 2003), including *Aedes aegypti,* a highly anthropophilic mosquito responsible for transmission of dengue and yellow fever around the world. Limited information is available about ecdysis in *A. aegypti*, especially with regard to larval and pupal ecdyses. In this work, we use *A. aegypti* as model disease vector to evaluate the molecular regulation of the ecdysis behavioral sequence.

☆ The nucleotide sequences reported in this paper have been deposited in the GenBank database and have the following Accession Nos. AeaETH gene (DQ864499), AeaETHR-A (DQ864500), AeaETHR-B (DQ864501).

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2. Materials and methods

2.1. Experimental animals and behavior observation

Mosquitoes (*A. aegypti*) were raised at 24 °C and fed a standard diet (Lea, 1964). For behavior observations and recordings, one to four living mosquitoes were positioned in a drop of water on a slide glass and observed with a compound microscope, which was connected to a Sony CCD camera. Behaviors recorded on videotape were analyzed and edited with Adobe Premiere. Morphological markers were used to stage insects. To elicit premature ecdysis, 10 fmol of synthetic peptide (AeaETH1 or AeaETH2) was injected in a 50 nl volume of phosphate-buffered saline (PBS) into staged mosquitoes using a glass pipette driven by a nano-injector. Water was removed during injection, and the mosquitoes were placed back into water immediately after injection. Phosphate-buffered saline (PBS) used as a negative control induced no ecdysis behavior under these conditions.

2.2. Immunohistochemistry and microscopy

Immunohistochemical identification of Inka cells was performed using a rabbit antiserum against *M. sexta* PETH (MasPETH) following procedures described previously (Zitnan et al., 1999). The tracheal system of staged mosquitoes was dissected in fly saline (in mM: 140 NaCl, 5 KCl, 1 $MgCl_2$, 5 $CaCl_2$, 4 $NaHCO_3$, 5 HEPES, pH 7.2) and fixed in 4% paraformaldehyde in 0.01 M PBS (pH 7.4) for 1–4 h at room temperature. After washing with PBS-1% Triton X-100 (PBST), tissues were blocked with 5% normal goat serum and incubated with rabbit anti-MasPETH (1:1000) for 2 days. Tissues were washed with PBST and incubated overnight at 4 °C with goat anti-rabbit whole IgG labeled with Alexa 555 (1:1000, Molecular Probes, Invitrogen, USA), washed sequentially in PBS, 30%, 50%, 70% and 90% glycerol diluted in PBS, and finally mounted in glycerol. DAPI staining was conducted in 5 ng/ml of PBST for 5 min. Labeled specimens were observed with a Zeiss Model 510 confocal laser microscope. When negative controls were performed by omitting primary antibodies, no immunolabeling was observed.

2.3. Isolation, identification and synthesis of AeaETHs

Heads and guts were removed from 400 pharate 4th instar larvae of *A. aegypti* and discarded. Remaining tissues were washed in fly saline and frozen at −70 °C until further use. Subsequently, tissues were heated to 90 °C for 5 min, homogenized in fly saline (100 μl per 50 animals), and centrifuged for 10 min at 10,000*g*. Supernatants were fractionated by reversed-phase HPLC (RP-HPLC; Rainin Instruments, Woburn, MA, USA) with a Microsorb C4 analytical column (4.6 × 250 mm, 5 μm particle size). Fractionation was accomplished using a linear gradient of acetonitrile (10–40% in 30 min and 40–80% in 15 min) in constant 0.1% trifluoroacetic acid (TFA) in water. Each HPLC fraction was dried and re-suspended in 500 μl of PBS, and 50 μl was used for enzyme immunoassay (EIA), which was performed with antiserum raised against PETH as described previously (Zitnanova et al., 2001). EIA-positive fractions were subjected to a second round of RP-HPLC using a Microsorb C18 analytical column (4.6 × 250 mm; 5 μm) with a linear gradient of acetonitrile (20–45% in 50 min) and constant 0.1% TFA in water. Fractions were screened again by EIA as described above, and those showing positive results were analyzed using Matrix-Assisted Laser Desorption Ionisation-Time of Flight Mass Spectrometry (MALDI-TOF MS; Voyager, USA) and sequenced using MALDI-TOF MS/MS in the Institute of Integrative Genome Biology Core Instrumentation Facility at UC Riverside.

Deduced amino acid sequences of AeaETHs were used for BLAST searching at the National Center for Biotechnology Information (NCBI) to identify the ETH gene in *A. aegypti*. Ambiguities in AeaETH sequence assignments made by MS/MS were resolved by analysis of the precursor nucleotide sequence.

AeaETHs were synthesized using an automated solid-phase peptide synthesizer (Applied Biosystems, USA Model 433) based on Fmoc-chemistry. Identities of native and synthetic peptides were confirmed by HPLC co-elution together with MALDI-TOF MS and MS/MS.

2.4. Cloning of AeaETH receptors

cDNA was synthesized from total RNA extracted from *A. aegypti* 4th instar larvae using the SuperScript II First-Strand Synthesis System for reverse transcription PCR (RT-PCR; Invitrogen) as described previously (Dai et al., 2007). Degenerate RT-PCR was performed with cDNA as template, Aea-df1 (5′-GNGTIGTIGGNA AYGTNATG-3′) and Aea-dr1 (5′-ARRTTRTAIARIATNGGRTT-3′) as forward and reverse degenerate primers designed for the conserved amino acid sequence region (VVGNVMV and NPILYNL) based on the known *D. melanogaster* ETH receptors (CG5911; GenBank Accession No. AY220741 and AY220742). Nested degenerate RT-PCR used the first round PCR product (1:10 diluted in water) as template, Aea-df2 (5′-GAIMGITAYTAYGCNATHTG-3′; ERYYAIC) and Aea-dr2 (5′-SWIACIACIGCNACNARCAT-3′; MLVAVVS) as forward and reverse primers. Products were cloned into the pGEM-T easy Vector system (Promega, USA) and sequenced and analyzed with Sequencher 4.1 and Vector NTI software. The whole cDNA sequence of AeaETHR-B was completed using 3′- and 5′-rapid amplification of cDNA ends (3′- and 5′-RACE). In order to obtain the cDNA of the AeaETHR-A subtype, genomic DNA extracted from 4th instar larvae of *A. aegypti* was used as template, Aea-df3 (5′-GTIYTNTAYGGIATHATHGC-3′; VLYGIIA) and Aea-dr3 (5′-ATIGGRTTCATIGCNSWRTT-3′; NSAMNPI) for the first round PCR, and Aea-df4 (5′-CARGTIGTIYTNATGYTNGG-3′; QVVLMLG) and Aea-dr4 (5′-ARCATDATICKRCARAARTA; YFCRIML) for the nested PCR. The full length cDNA of AeaETHR-A was obtained using 3′- and 5′-RACE methods as described above. The entire open reading frames (ORFs) of AeaETHR-A, B overlapped by 3′- and 5′-RACE results were confirmed by RT-PCR using Aea-f1 (5′-CAACGAGTCCTACAGCGAAA-3′) and Aea-r1 (5′-AAAAAAGCCTACTCCTTTGC-3′) for AeaETHR-A, and Aea-f1 and Aea-r2 (5′-AGACACCCCGCAGCAACC CC-3′) for AeaETHR-B. ORFs of both subtypes were cloned into the pcDNA3.1 vector (Invitrogen) for subsequent expression in Chinese hamster ovary (CHO) cells.

2.5. Phylogenetic analysis

The ClustalW default option in Mega4 (v.4.0.2) was used for multiple sequence alignment. Phylogenetic analysis was performed using the aligned sequences, with pairwise deletion for gap/missing data option according to a PAM matrix model. A neighbor-joining tree was constructed with 500 bootstrapping replicates. Other methods, UPGMA and minimum evolution trees, showed an identical tree topology.

2.6. Cell line expression and functional analysis of AeaETHRs

Wild type CHO cell lines (CHO-K1) were transiently transfected with codon-optimized aequorin and AeaETHR-A or AeaETHR-B as described previously (Park et al., 2003; Vernon and Printen, 2002). FuGene6 (Roche Molecular Biochemicals) mixed with DNA (3:1) was used for transfection following the manufacturer's protocol. Transfected cells were incubated for 1 day at 37 °C in 5% CO_2. Coelenterazine h (Molecular Probes, Invitrogen, USA) was added