Published in final edited form as:

Gen Comp Endocrinol. 2009 May 15; 162(1): 43–51. doi:10.1016/j.ygcen.2009.03.004.

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.

Keywords

Ecdysis triggering hormone; Eclosion; Ecdysteroid; Receptor; Behavior; Phylogeny; HPLC; Mass spectrometry; Gene expression

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).

[☆]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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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.

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 MgCl₂, 5 CaCl₂, 4 NaHCO₃, 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 10,000g. 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'-GNGTIGTIGGNAAYGTNATG-3') and Aea-dr1 (5'-ARRTTRTAIARIATNGGRIT-3') as forward and reverse degenerate primers designed for the conserved amino acid sequence region (VVGNV MV 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'-ATIGGRTTCATIGCNSWRIT-3'; NSAMNPI) for the first round PCR, and Aea-df4 (5'-CARGTIGTIYTNTATGYTNGG-3'; QVVLMLG) and Aeadr4 (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'-AGACACCCCGCAGCAACCCC-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% CO₂. Coelenterazine h (Molecular Probes, Invitrogen, USA) was added into cell suspensions 3–5 h before starting functional assays. Luminescence assays were performed using a Beckman model LD400 Luminometer, following the procedures described previously (Park et al., 2003; Vernon and Printen, 2002). The following peptides were used: AeaETH1, AeaETH2, DrmETH1, DrmETH2, MasPETH, MasETH, MasETHacid, DrmCAP_{2b-1}, DrmCAP_{2b-2}, SCP_b, Hug-2, NMU-8, Ghrelin, FMRFamide and corazonin. Buffer only was used as a negative control.

2.7. Determination of ecdysteroid levels by enzyme immunoassay (EIA)

The average developmental time from the beginning of the 4th instar to pupation is 62 h at 24 °C. We collected animals at 40, 36, 28, 26, 20, 16, 14, 12, 10 or 8 h before ecdysis for (1) ecdysteroid determinations, (2) analysis of ecdysis behavior in response to ETH injection, and (3) determination of ETHR transcript levels by q-RT-PCR. Each time point has six animals repeated for each experiment. Beginning at 12 h prior to pupation, stage-specific morphological markers become visible.

Total ecdysteroid titers of staged mosquitoes were measured using EIA as described previously (Kingan, 1989). Staged 4th instar larva were collected and heated at 80 °C for 5 min, electrically homogenized and centrifuged for 10 min at 10,000g. Supernatants were analyzed by EIA using an ecdysteroid-directed antiserum diluted 1:300,000.

2.8. Quantitative RT-PCR

Staged 4th instar larvae were collected and immediately frozen and stored at –80 °C. Total RNA was extracted using TRIzol (Invitrogen, USA) following the manufacturer's protocol and genomic DNA was removed by treating with 1 U DNAase I (Invitrogen, USA) for 15 min at room temperature. 5 µg of total RNA was used for cDNA synthesis using SuperScript II First-Strand Synthesis System for RT-PCR (Invitrogen, USA) as described above. ETHR cDNA was quantified by performing real-time quantitative RT-PCR using QuantiTect SYBR Green PCR kit (Qiagen) and ABI PRISM 7700 (USA), with quantified plasmid with ETHR ORFs inserted as standard control. Forward and reverse primers (5'-GTCTGCACACCAACCGTACT-3' and 5'-GATATCGCCAGGATCGTCAG-3') were

designed to amplify 137 bps spanning exons 1 and 2. Quantitative PCR (25 μ l) contained 12.5 μ l of 2 $\times$ QuantiTect SYBR Green PCR Master Mix, 0.3 μ M of each primer, template DNA or water as negative control. PCR thermal cycler conditions were programmed as follows: 50 °C for 2 min, 95 °C for 15 min, 40 cycles of 94 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s.

3. Results

3.1. Inka cells in *A. aegypti*

Using a MasPETH antiserum, we immunolabeled Inka cells of pharate 4th instar larvae and found them to be located on the surface of lateral tracheal trunks (Fig. 1A). Inka cells exhibit strong PETH-like immunoreactivity (PETH-IR) when stained ~3 h prior to ecdysis. However, PETH-IR had disappeared when tissues were stained shortly after ecdysis (Fig. 1B) suggesting release of Inka cell peptides during ecdysis.

3.2. Identification of two ETH peptides from Inka cells of *A. aegypti*

To identify ETH peptides in *A. aegypti*, 400 pharate 4th instar larvae were fractionated by RP-HPLC. Fractions containing ETHs were screened by EIA (Fig. 2A and B). In the first round of HPLC, only fractions eluting between 36–39% acetonitrile in 0.1% TFA showed positive immunoreactivity to MasPETH antiserum. Total immunoreactivity of recovered peptides was estimated to be ~50 pmol/animal. This fraction was further separated by a second HPLC step, and two fractions (FII-44 and FII-48) eluting at 36% and 38% acetonitrile in 0.1% TFA, respectively, showed positive EIA results. Contents of these fractions were subjected to MALDI-TOF MS and MALDI-TOF MS/MS analyses (Fig. 2B). One peptide (2011.2 Da) in fraction FII-44 was sequenced as GDFENFFI/LK/QK/QSK/QSVPRI/L-NH₂, and the other (1933.1 Da) in fraction FII-48 was sequenced as DETPGFFI/LK/QLSK/QSVPRI/L-NH₂. Both of these peptides contain the conserved-PRXamide C-terminal amino acid motif found in previously characterized ETHs. Ambiguities in amino acid assignments, for example Ile/Leu and Gln/Lys were resolved by identification of the ETH precursor through BLAST analysis of the *A. aegypti* genome using the AeaETH peptide sequences. The nucleotide sequence encoding the *A. aegypti* ETH precursor (GenBank Accession No. DQ864499; Fig. 2C–E), encodes two ETH peptides, named here AeaETH1 (2011.2 Da) and AeaETH2 (1933.1 Da), based on their location in the ETH gene and homology with DrmETH1 and DrmETH2. ETHs in *A. aegypti* are highly similar and other insect ETHs share high homology (Fig. 2D), especially with the related mosquito *Anopheles gambiae* (only one to two amino acid variance in the N-terminal part of the peptides). The two *A. aegypti* ETH peptides were chemically synthesized and show identical elution time in the HPLC chromatograph, MS and MS/MS spectra (data not shown). Synthetic peptides were used for injection into staged mosquitoes and ETHR functional expression assay below (see below).

A putative ecdysone receptor response element (EcRE), AGCACAtgcaCGAGCT, with four nucleotides separating two imperfect inverted repeats, was identified 300 bps upstream of the open reading frame (ORF). Similar EcREs have been described in *D. melanogaster* (AGGTCAggttAGGTCA) and *A. gambiae* (AGGTCAatTCACCT) (Park et al., 1999; Zitnan et al., 2003).

3.3. Natural and AeaETH-induced ecdysis behaviors in larvae, pupae and adults

Natural ecdysis behaviors of *A. aegypti* were observed and recorded at the following transitions: larval ecdysis (3rd–4th instar), pupal ecdysis, and adult eclosion (Fig. 3A). Third instar larvae exhibit a large ballooned and transparent head immediately after ecdysis, which hardens and darkens several hours later (Christophers, 1960). About 6–8 h before ecdysis, the pharate 4th instar dark hairs become visible across the thoracic and abdominal segments of the pharate 4th instar larva. Upon pre-ecdysis initiation (defined as time = 0), larvae exhibit regular

contractions and extensions, each cycle lasting 1–2 s followed by quiescence for several seconds or longer. During pre-ecdysis, slight mechanical disturbances cause momentary cessation of the behavior, which is renewed within 30–60 s. Pre-ecdysis lasts ~5–6 min followed by ecdysis initiation. Once ecdysis starts, the animal becomes insensitive to touching or mechanical disturbances. During ecdysis, the mosquito larva performs rhythmic peristaltic contractions from posterior to anterior continuously for 30–60 s, upon which the old cuticle splits at “ecdysial seams” on the head and thoracic segments and is eventually shed.

Unlike larvae, pharate pupae display no black hairs across the body. However, one pair of transparent pupal trumpet rudiments on both sides of the prothorax appear about 12 h before ecdysis, and darken to become brown-black about 7–9 h before ecdysis. At this time, two black brushes also appear on the dorsal side of first abdominal segment. The pupal ecdysis behavioral sequence begins with pre-ecdysis lasting for ~10 min. During this phase, the animal alternates between anterior-posterior contractions lasting 1–2 s followed by rest (5–6 s or longer). Ecdysis begins with uninterrupted peristaltic contractions from posterior to anterior (1–2 s) and lasts about 1–3 min until the animal sheds its old cuticle completely.

Some 12 h prior to eclosion, the pharate adult body color becomes darkened. In contrast to the ecdysis sequences observed in previous stages, no obvious pre-ecdysis contractions are observed prior to initiation of eclosion, characterized by rhythmic peristaltic contractions. About 1–2 min after rhythmic contractions begin, the old cuticle splits at the head and thoracic segments and the adult mosquito emerges 3–5 min later.

Following characterization of natural ecdysis behaviors, we asked whether injection of synthetic AeaETH1 and AeaETH2 could induce premature ecdysis or eclosion behavior. Both peptides induced premature ecdysis behaviors, and were equipotent in doing so. We focused on pupal ecdysis, because pharate pupae have more obvious morphological markers for precise developmental staging (Fig. 3B). Upon appearance of pupal trumpet rudiments (about 9–12 hours before ecdysis), 10 fmol (50 nl of 200 nM) of either AeaETH1 or AeaETH2 was injected into the abdomen. After a latency of ~8–9 min, during which no pre-ecdysis contractions were evident, rhythmic peristaltic ecdysis contractions from posterior to anterior (11 out of 12 animals) were observed. Ecdysis contractions lasted ~10 min and then ceased without the old cuticle being shed. About 12 h after ETH-induced premature ecdysis behavior was observed, animals initiated the natural ecdysis sequence and shed the old cuticle (see Fig. 3).

Different results were obtained if AeaETHs were injected closer to natural ecdysis. Injections administered less than 4 h before ecdysis resulted no obvious pre-ecdysis behavior, but persistent ecdysis contractions lasting ~40 min were observed. Treated animals were unable to shed the old cuticle and after contractions gradually subsided, death ensued.

3.4. Two subtypes of AeaETHRs arise through alternative splicing of 3'-exons

Based on the assumption that insect ETHRs are conserved of primary sequences, we cloned two subtypes of AeaETHRs using degenerate PCR, 3'- and 5'-RACE and RT-PCR. Two pairs of degenerate primers designed according to DroETHR (CG5911) were used for first and second rounds of nested PCR. A 250 bp band was obtained and sequenced, and showed high homology to insect ETHRs reported previously (Park et al., 2003). Subsequent 3'- and 5'-RACE yielded a cDNA fragment containing a 1761 bp open reading frame (ORF). It was named A. *aegypti* ETH receptor subtype B (AeaETHR-B) based on its high homology with DrmETHR-B (Fig. 4A). However, repeated attempts using the cDNA as template for degenerate PCR failed to amplify the homolog of DrmETHR-A. We therefore adopted an alternative strategy of using genomic DNA as template and two pairs of degenerate primers designed according to the sequence of DrmETHR-A for degenerate PCR. A 165 bps band was obtained, which showed a sequence distinct from that of AeaETHR-B. By overlapping the 3'- and 5'-RACE

products, we obtained a 1701 bps ORF, and named it *A. aegypti* ETH receptor subtype A (AeaETHR-A), which shared high homology with other insect ETHR-As (Fig. 4A).

3.5. AeaETHRs are sensitive and selective for ETHs

We developed flash luminescence assays to monitor AeaETHR-A or AeaETHR-B activation by a variety of peptide ligands. The assay, involving transient co-expression of receptor and codon-optimized aequorin in CHO-K1 cells, is similar to those described previously (Kim et al., 2004, 2006b; Le Poul et al., 2002; Park et al., 2003). Receptor activation mobilizes calcium from intracellular stores, leading to activation of an aequorin-coelenterazine complex to produce luminescence, which is proportional to the amount of calcium mobilized. Concentration-response curves generated by exposure of mosquito, fly, and moth ETHs showed that both AeaETHR-A and AeaETHR-B are most sensitive to dipteran ETH peptides, with EC₅₀ values in the low nanomolar range. AeaETHR-A is most sensitive to AeaETH1 (EC₅₀ ~ 4 nM) and DrmETH1 (EC₅₀ ~ 8 nM). Other ETH peptides also are active, according to the following rank order from highest to lowest potency: AeaETH1 > DrmETH1 > AeaETH2 (~23 nM) > MasETH (~61 nM) > DrmETH2 (~97 nM). PETH was much less active, with an EC₅₀ ~ 764 nM. On the other hand, AeaETHR-B is most sensitive to DrmETH1 (EC₅₀ ~ 10 nM). AeaETH1 (~20 nM) and AeaETH2 (~29 nM) are essentially equipotent and only slightly less active than DrmETH1. The rank order of ligand potency for AeaETHR-B is DrmETH1 > AeaETH2 > AeaETH1 > DrmETH2 (~48 nM) > MasETH (EC₅₀ ~ 112 nM). PETH shows an apparent low affinity for AeaETHR-B, eliciting only a slight response at 10 μM, the highest concentration tested. Likewise, MasETHacid elicits only slight responses from both receptors when tested at 10 μM.

An assortment of peptides with the-PRXamide C-terminal amino acid motif show significant activity when tested at 1 and 10 μM. These include DrmCAP_{2b-1}, DrmCAP_{2b-2}, and DrmCAP_{2b-3}, and the molluscan cardioacceleratory peptide SCP_B. In fact, SCP_B was the most active of the peptides in this group (Fig. 5C and D). Several peptides with related sequences, including Hug-2, NMU-8, ghrelin, FMRFamide, and corazonin, were inactive when tested at 10 μM.

3.6. Expression of ETHRs and onset of behavioral sensitivity to ETHs coincides with elevation of ecdysteroids

We injected AeaETHs 1 day prior to natural pupal ecdysis and found that animals were insensitive to the peptides. Previous studies in *M. sexta* showed that behavioral sensitivity to ETHs appears coincident with elevated steroid levels at the onset of the molt (Kim et al., 2006b; Zitnan et al., 1999). We therefore investigated the relationship between ecdysteroid levels, ETHR expression, and behavioral sensitivity to AeaETH. For determination of ecdysteroid levels, we performed enzyme immunoassays. Quantitative real-time RT-PCR was used to quantify ETHRs transcripts and ecdysis behaviors were observed following ETH injection.

Rising ecdysteroid levels were first detected ~25 h prior to pupation, reaching peak levels (30 fmol/mg body weight) about 12 h prior to ecdysis (Fig. 6). AeaETHR transcripts also become detectable ~25 h prior to pupation, and reach peak levels (3.5 μg/mg body weight) at ~12 h. Behavioral sensitivity to injected AeaETHs arose about 12 h prior to ecdysis. At this time, about 20% of injected animals responded to AeaETH1 injection (10 fmol). At ~10 h, more than 80% animals showed premature ecdysis behavior in response to ETH peptide injection. These data suggest that ecdysteroids induce expression of ETH receptors and behavioral sensitivity to ETHs.

4. Discussion

4.1. ETHs and ETH receptors in *A. aegypti* are highly conserved

Inka cells and ETH-like peptides have been described in a wide range of insect orders on the basis of immunohistochemical staining and across-species bioassays (Zitnan et al., 2003). However, definitive descriptions of specific ligands and their receptors, along with regulatory elements influencing their expression have been accomplished in only a few species: The moths *M. sexta* and *Bombyx mori* (Kim et al., 2006b; Zitnan et al., 2002, 1999) and the fruit fly, *D. melanogaster* (Iversen et al., 2002; Park et al., 2003, 1999). We have characterized two bioactive ETH peptides isolated from the mosquito *A. aegypti*, AeaETH1 and AeaETH2, and their cognate receptors AeaETHR-A and AeaETHR-B. Behavioral studies show that both AeaETHs trigger ecdysis behavioral sequences in larvae, pupae and adult mosquitos when injected into larvae prematurely.

AeaETHs were isolated from tracheal tissues by HPLC and sequenced *de novo* using mass spectrometry. Confirmation of the sequences were obtained by comparison to peptide sequences embedded in the precursor protein deduced from the *A. aegypti* genome. Comparison of the MS-derived AeaETH peptide sequences with those deduced from the precursor served two purposes. First, ambiguities in amino acid assignments (e.g., I/L; K/Q) at certain positions were resolved. Second, the MS-derived sequences confirmed locations of processing sites in the pre-propeptide precursor. This was especially useful for pinpointing the cleavage site between the signal sequence and the N-terminus of AeaETH1. Both AeaETH peptides share high sequence similarity with other known ETH peptides identified in moths and flies and the organization of the *A. aegypti* cDNA precursor is likewise similar. In particular, we identified a putative ecdysone receptor response element about 300 bp upstream of the methionine start site, which is likely involved in induction of the ETH gene at molt onset.

Unlike PETH and ETH in moths, whose N-terminal amino acid sequences are variable, AeaETHs are 17-mers, as are those of *A. gambiae*. In addition, the sequences of the mosquito peptides are highly conserved, differing at only one position in the C-terminal 12-mer. This may account for the fact that AeaETH1 and AeaETH2 are virtually equipotent as activators of ETH receptors and as chemical triggers of the mosquito ecdysis behavioral sequence in injection bioassay experiments. Surprisingly, we found that both AeaETHs trigger pupal ecdysis behavior, in the absence of pre-ecdysis behavior.

This contrasts with the properties of PETH and ETH action in moth larvae, where clear differences are observed in their biological actions. PETH initiates pre-ecdysis I only, whereas ETH alone is capable of inducing pre-ecdysis I, pre-ecdysis II and ecdysis (Zitnan et al., 1999). In *Drosophila* larvae, DrmETH1 and DrmETH2 also have different properties. DrmETH2 is ~10-fold less active than DrmETH1 when tested on CG5911a, whereas the two peptides activate CG5911b with similar potency (Park et al., 2003). Behavioral differences also are observed. Treatment of fly larvae with DrmETH1 induces the entire ecdysis behavioral sequence, whereas DrmETH2-treated larvae perform ecdysis in the absence of preecdysis (Park et al., 2002). This is reminiscent of the action of AeaETHs on pupal ecdysis.

Thus, multiple ETH peptides appear to play different behavioral roles depending on the species. The functional significance of these differences remains largely obscure. Additional work is needed to ascertain what types of selective pressures have operated on the ETH gene during the course of evolution. Perhaps the significance of multiple ETHs will come into clearer focus as more species are examined and possible stage-specific (larva, pupa, adult) processing of these precursors is analyzed in more comprehensive fashion.

Two distinct ETHR subtypes (AeaETHR-A and AeaETHR-B) in *A. aegypti* are encoded by a single gene via alternative splicing of two 3'-exons. These exons encode the protein sequence from the 4th transmembrane segment to the C-terminus, accounting for ~75% of the receptor protein. Although sequence similarity among ETHRs is high, especially among the 7-transmembrane segments of the protein, phylogenetic analysis shows that mosquito A- and B-receptor subtypes segregate into separate clades (Fig. 4B). The evolutionary divergence of the receptor subtypes suggests they have been subjected to different selective pressures, possibly related to their different functions. Consistent with this idea, ETHR-A and ETHR-B are expressed in non-overlapping populations of central neurons in *Drosophila* and *M. sexta* (Kim et al., 2006a,b). Further work is needed to evaluate the functional significance of the two receptor subtypes.

We have found that activation of AeaETHR-A and AeaETHR-B expressed in CHO-K1 cells mobilizes intracellular calcium, findings consistent with previous accounts of ETHRs in *Drosophila* and *M. sexta* (Iversen et al., 2002; Kim et al., 2006a; Park et al., 2003). Pharmacological profiling of AeaETHR-A and AeaETHR-B indicates that both are sensitive and selective for ETH peptides from mosquitoes, moths and flies compared to other—PRXamide peptides such as CAP2Bs and SCP_B from mollusks. Not surprisingly, dipteran ETHs are generally more potent ligands than those of the moth, *M. sexta*. However while MasETH is within an order of magnitude as potent as the best dipteran ligands, MasPETH is >100-fold less active on AeaETHR-A and is virtually devoid of activity when tested on AeaETHR-B. This highlights a unique feature of the mosquito ETHR binding pocket, since MasPETH is much closer to the potencies of other ETH ligands when tested on *Drosophila* and *Manduca* receptors. It may be that the proline at position five of MasPETH, which is lacking in all the other known ETHs, introduces a distinctive bend in the primary structure that is less well tolerated by mosquito ETHRs.

4.2. Receptor expression and behavioral competence coincide with rising ecdysteroid levels

Previous work has demonstrated that ETHs act directly on the central nervous system to trigger the ecdysis behavioral sequence in *M. sexta* (Zitnan et al., 1999; Zitnanova et al., 2001). However, behavioral sensitivity to ETH injection arises only during pharate stages, coincident with ecdysteroid-induced molt onset. In the present work dealing with *A. aegypti*, we also found a close correspondence between behavioral responsiveness to ETH injection and rising ecdysteroid levels. This occurs about 14 h prior to pupation. Shortly after ecdysteroid levels peak, 60–80% of treated pharate pupae show sensitivity to ETH injection. This suggests that behavioral responsiveness of insects to ETH depends upon rising ecdysteroid levels. Confirmation of this came in moth with the demonstration that pre-treatment of early 5th instar *M. sexta* with 20E induces behavioral sensitivity (Zitnanova et al., 2001). Furthermore, pre-treatment of the isolated CNS with 20E induces sensitivity to ETH applied *in vitro* (Zitnanova et al., 2001).

The mechanism by which 20E induces behavioral sensitivity to ETH is likely via induction of ETHR expression. We have shown here in *A. aegypti* that levels of ETHR transcript rise in register with steroid levels, consistent with data reported recently in *M. sexta* (Kim et al., 2006a). Molecular mechanisms underlying steroid regulation of the ETHR remain unclear, but are under investigation.

Steroid levels regulate not only ETHR expression, but also synthesis of ETHs as well as release of these peptides from Inka cells. A sharp increase in ETH levels in Inka cells of *M. sexta* occurs immediately after steroids rise at molt initiation, and injection of ecdysteroid into freshly molted animals induces production of ETHs (Zitnanova et al., 2001). The presence of an ecdysteroid response element (EcRE) in the promotor region of several ETH genes suggests direct regulation of the ETH gene by EcR (Park et al., 1999; Zitnan et al., 2002, 2003). On the

other hand, declining steroid levels are required for secretory competence of Inka cells (Kingan and Adams, 2000; Kingan et al., 1997). Taken together, genes encoding ETH and ETHR appear to be under tight regulation by 20E. Further studies are needed to understand the mechanism by which ETH signaling initiates and schedules stereotypic ecdysis behavioral sequences in insects. Furthermore, ETH signaling is vital to successful ecdysis in flies and moths (Dai et al., 2008; Park et al., 2002), and further work on this system in mosquitos may lead to improved approaches for vector control in the future.

Acknowledgments

We thank Alexander Raikhel for providing mosquito eggs, Ingrid Zitnanová for help with enzyme immunoassays, Dusan Zitnan for advice on immunohistochemistry, and Timothy Kingan for helpful discussions. We are especially grateful to Yoonseong Park for help with phylogenetic analysis. This work was supported by NIH Grant GM67310.

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Fig. 1. Immunohistochemical labeling of Inka cells of *A. aegypti* using an antiserum raised against *M. sexta* PETH. Intense immunoreactivity is observed in Inka cells of 4th instar larvae at 3 h before ecdysis (A). Staining disappears just after ecdysis (B). Scale bar, 50 μ m.

Fig. 2. Identification of two *A. aegypti* ETH peptides by HPLC, enzyme immunoassay (EIA) and mass spectrometry. (A) Extract of *A. aegypti* pharate 4th instar larvae was HPLC-fractionated using a Microsorb C₄ analytical column (4.6 × 250 mm; 5 μm particle size) with a linear gradient of acetonitrile (10–40% in 30 min and 40–80% in 15 min) in constant 0.1% TFA. All fractions were subjected to EIA using an antiserum against PETH. The shaded histogram bars depict PETH-like immunoactivity (PETH-IR) of each fraction. (B) Fractions eluting at 36–39% acetonitrile showing positive EIA results were further fractionated with a Microsorb C₁₈ analytical column (4.6 × 250 mm; 5 μm) with a linear gradient of acetonitrile (20–45% in 50 min) in constant 0.1% TFA. Fractions (FII-44, FII-48) eluted at 36% and 38% acetonitrile in

0.1% TFA contained AeaETH2 and AeaETH1, respectively. The peptides were sequenced using MALDI-MS and MS/MS. (C) Sequencing of AeaETH1 using MALDI-TOF MS/MS. The amino acid sequence shown in the inset was assigned from b and y ion series. Ambiguities in the sequence (Ile/Leu and Gln/Lys) were resolved by analysis of deduced amino acid sequences from the ETH gene (see text for details). (D) The ETH gene and deduced amino acid sequences of AeaETH1 and AeaETH2 obtained from the *A. aegypti* genome database. The open reading frame (ORF) of the ETH gene is depicted in upper-case letters, and non-coding sequences are in lower-case letters. The putative ecdysone receptor response element (EcRE) in the promoter region is underlined, and the intron donor and acceptor motifs (gt, cag) are italicized. The poly-A signal (aataaa) is italicized and underlined. The deduced amino acid sequence of the precursor protein is shown in upper-case letters, and the fully processed peptides AeaETH1 and AeaETH2 are boxed. (E) Amino acid sequence alignment of insect ETHs.

Fig. 3.

Natural and ETH-induced ecdysis behavior sequence of *A. aegypti*. (A) Stereotypic ecdysis behaviors occur at predictable times during development. Larval ecdysis (3rd–4th instar), pupal ecdysis and adult eclosion are described. In each case, initiation of the ecdysis behavioral sequence is indicated as time zero. Morphological markers, which help to pinpoint the developmental stage prior to ecdysis are indicated. (B) Injection of synthetic AeaETH1 or AeaETH2 at either -12 h or -4 h induces premature ecdysis behavior. Treatment of pharate pupa at -12 h induced premature ecdysis with a latency of 8–9 min, which lasted ~10 min and stopped. The animal subsequently went into natural ecdysis and shed the old cuticle. However, animals injected at -4 h performed ecdysis behavior for more than 40 min and were not able to initiate the natural ecdysis. These animals died.

Fig. 4. Molecular characterization of AeaETH receptors in *A. aegypti*. (A) Genomic structure of ETHRs in *A. aegypti*, *D. melanogaster* (Park et al., 2003) and *M. sexta* (Kim et al., 2006b). (B) Phylogenetic tree depicting evolutionary relationships of ETH receptors in *Aedes aegypti*, *Anopheles gambiae*, and *Drosophila melanogaster* and the moth *Manduca sexta*.

Fig. 5. Pharmacological profiles of AeaETHRs expressed heterologously in CHO-K1 cells. (A) Concentration-response relationships for peptides tested on AeaETHR-A. (B) Concentration-response relationships for peptides tested on AeaETHR-B. Insets in each plot show EC₅₀ values for each ligand. (C and D): Activity of peptides having C-terminal amino acid motifs similar to those of ETHs.

Fig. 6.

Relationship of AeaETHR expression, behavioral sensitivity to ETH, and ecdysteroid levels in pharate pupae. Pharate pupae collected between -38 and -7 h relative to ecdysis (time 0) were used to measure the ecdysteroid level by performing enzyme immunoassay (EIA). ETH injection assays were used to determine behavioral sensitivity. Relative levels of ETHR transcript were determined by quantitative RT-PCR. At least 6 individuals were used for each time point.